ENDOCRINOLOGY

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Still, however, the coincidence is so frequent and remarkable, that one can scarcely avoid suspecting that the thyroid gland, of which no use whatever has hitherto been hinted at by physiologists, is intended in part to serve as a diverticulum in order to avert from the brain a part of the blood, which, urged with too great force by various causes, might disorder or destroy the functions of that important organ.

—Caleb Hillier Parry,
Elements of Pathology and Therapeutics, Vol. 2. 1825.

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Errata, Volume 28

Page 239, line 12. For figure 1A *read* figure 1B.

Page 239, line 19. For figure 1B *read* figure 1A.

Page 285, paragraph 9. For $\sigma\sqrt{N-1}$ *read* $\sigma/\sqrt{N-1}$.

Page 290, paragraph 5, line 3. For P=0<.002 *read* P= <0.002.

Page 296, table 3, column "Difference," second line. For ±23.1+4.6 *read* +23.1±4.6.

Page 300, line 10. For 4.0±24.9 *read* 7.40±24.9.

Pages 341–344 February, and 341–344 March, bear duplicate numbers and are indexed 341a–344a (*February*) *and* 341b–344b (*March*).

Page 341a, line 12. For unoperated miles *read* unoperated males.

Page 342a, line 7. For mammary glands *read* mammary gland.

Page 343, lines 23 and 24. For ovary is sufficient prevent *read* ovary is sufficient to prevent.

Page 414. Insert reference to footnote at end of second paragraph and insert at bottom of page the footnote
Blood stood a week on ice before analysis and thyroxin may have combined with protein. When analyzed immediately recovery is not complete.

Page 414, line 2, paragraph 5. For glands considered justification *read* glands is considered justification.

Page 415, paragraph 6. Delete flasks are placed in a shaking or rotating machine for 1 hour, after which the.

Pages 416, 417, 418. For Thyroxin iodine *in text and in tables 5 and 7 read* thyroid iodine.

Page 417, line 21. For thyroxin *read* thyroid.

Page 418, line 6. For Thyroxin *read* thyroid iodine.

ENDOCRINOLOGY

PUBLISHED FOR

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VOLUME 28 JANUARY, 1941 NUMBER 1

INNERVATION OF THE HYPOPHYSIS OF THE RABBIT AND RAT[1]

CHANDLER McC. BROOKS AND I. GERSH

From the Departments of Physiology and Anatomy, The Johns Hopkins University

BALTIMORE, MARYLAND

PHYSIOLOGICAL EVIDENCE suggests that in some mammals certain hypophysial functions are under direct nervous control. An abundant literature clearly indicates the operation of such a factor in the secretion of an antidiuretic substance by the neurohypophysis of several species (1, 2, 3). Likewise there is evidence that in the rabbit and rat certain functions of the anterior lobe are subject to neural regulation (4, 5, 6). The anatomical basis for a functional innervation of the hypophysis has been reviewed recently by Rasmussen (7). The work described in this paper was undertaken to investigate more thoroughly the nerve fiber pattern in the pituitary gland of the rabbit and the rat.

PROCEDURES

The hypothalamus with hypophysis attached was prepared by the pyridine-silver method and sectioned serially at 10 μ. With few exceptions, the ventral surface was protected by covering it before fixation with a thin layer of brain tissue. Detailed studies were made only of those preparations in which the nerve fibers of the protective layer and the hypothalamus were well impregnated with silver.

Young animals were used throughout this study. Eight hypophyses were obtained from normal rabbits which were from 4 to 6 days old when killed. Bilateral superior cervical ganglionectomy was performed in 4 animals which were killed 5 days later when 9 days old. Five additional rabbits were subjected to partial or complete stalk section by electrolytic lesions made with a unipolar electrode inserted by a simple stereotaxic instrument through a small opening in the calvarium; the large indifferent electrode was placed on the abdomen. These young rabbits were nursed and cared for normally by their mothers until killed. Due to the close approximation of cell bodies and nerve fibers concerned it was felt that the times allowed (3 to 8 days) were adequate for the complete degeneration of the fibers peripheral to the lesion. With the exception of 2 adults, most of the 14 normal rats were 3 weeks old when the operations were performed. The superior cervical ganglion of each side was removed in 3 young rats which were killed 10 days later. In the 2 adult rats and in 4 young animals the hypophysial stalk was exposed through a temporal approach and an attempt made to sever it. In only one case was the stalk completely cut, but in another instance transection was almost complete.

Received for publication July 19, 1940.

[1] A preliminary report of a part of this work was published in the *Anat. Rec. Suppl.* 3, 70: 10. 1938.

This investigation was aided by a grant (to Dr. Philip Bard) from the Committee for Research in Problems of Sex, National Research Council.

OBSERVATIONS

Normal rabbits. Numerous nerve fibers pass down the hypophysial stalk to the infundibular process. Most of these enter through the anterior part of the median eminence; those lying in the posterior part are relatively few in number. The fibers received from both sources are distributed primarily throughout the infundibular process so as to form a dense, complex interweaving mesh of the type described by Cajal for the mouse. No clear nerve endings were observed in the infundibular process. A very small number of fibers from the infundibular stem directly enter the pars intermedia, but most of the nerve fibers in this part of the hypophysis appear to be derived from isolated, scattered fibers or bundles which emerge from the infundibular process at various points along the boundary between the two parts of the posterior lobe. In one case fibers could be traced along a small capsular artery directly into the pars intermedia. Delicate nerve fibers were visible in all parts of the pars intermedia. An average of about fifty segments of nerves were seen in a 10 μ section cut through this portion of the hypophysis at its greatest dimension. Nerve fibers were found unevenly distributed in the septa as well as between individual cells of all parts of the pars intermedia but they were much more numerous around the periphery of the residual lumen of Rathke's pouch. In this region the direction of the fibers was always from the infundibular process to the anterior lobe. Elsewhere, the nerve fibers coursed in almost any direction. It is apparent that they enter the anterior lobe from three sources: the infundibular stem, the infundibular process by way of the pars intermedia, and the capsular arteries. Thirty or more segments of nerve fibers could be seen in a single 10 μ section cut through the thickest portion of the pars distalis. They were present in all parts of the section but tended to be more numerous at its anterior tip.

Rabbits with superior cervical ganglionectomies. In the neurohypophysis and pars intermedia the distribution of nerve fibers could not be distinguished from that seen in normal animals. In contrast to this, there was an apparent reduction in the number of nerve fibers present in the anterior lobe. All the hypophyses contained bundles of nerve fibers which were associated with or incorporated in the walls of the capsular arteries.

Stalk-cut rabbits. In this series the results of the operations were not identical. Hence the main features of each preparation will be described separately.

(*a*) *Partial stalk section* (E 2). This animal was operated on when 4 days old; killed 7 days later. There was a small unilateral lesion in the median eminence. The infundibular stem had been partially severed close to the posterior lobe where there was a large swelling and deposition of scar tissue. Only a few fibers in the anterior portion of the stalk continued down into the infundibular process. Immediately proximal to the lesion, each section of the stalk contained as many as ten nerve fibers. In practically all sections of the infundibular process not more than one nerve fiber could be seen. The majority of the sections through the pars intermedia were devoid of nerve fibers, a few showed one, a single section was unique in that it contained as many as 6. Many sections through the anterior lobe contained 5 to 10 nerve fibers; 2 sections, however, showed about 15.

(*b*) *Large hypothalamic lesion* (F 3). This rabbit was operated on when 3 days old; killed 3 days later. A few nerve fibers passed down the periphery of the stalk. The majority of the fibers in the infundibular process were confined to its proximal part; the remaining ones were distributed chiefly adjacent to the pars intermedia. Two nerve fibers were seen extending between the infundibular process and the pars intermedia. Nerve fibers in the adenohypophysis were greatly reduced in number as compared with the normal. In many sections of the anterior lobe only one or two nerve fibers could be seen; the greatest number visible in any one section was 6.

(c) *Complete stalk section* (F 2). There was some injury to the median eminence and to the hypothalamus anterior to this along the mid-line. This rabbit was operated on when 3 days old; killed 3 days later. The tip of the anterior lobe was injured. The pars nervosa was shrunken and contained approximately 10 nerve fibers per section. No nerve fibers were seen in the pars intermedia. In the anterior lobe an average of 4 nerve fibers per section was found; 9 was the greatest number observed in any one section.

(d) *Complete stalk section with a small lesion in the median eminence* (E 5). This rabbit was operated on when 4 days old; killed 8 days later. Sections of the shrunken pars nervosa contained a few nerve fibers varying in number from 0 to 6 with an average of 2. In the pars intermedia never more than 3 fibers were visible in any one section; the average was one. The number of nerve fibers per section found in the anterior lobe varied from none (one case) to 10 (two cases). The majority of sections contained approximately 4 fibers.

(e) *Complete stalk section* (E 6). This rabbit was operated on when 4 days old; killed 8 days later. The lesion not only separated the infundibular stalk from its attachment to the gland but also cut the anterior lobe in two. The shrunken pars nervosa showed an average of 1.5 nerve fibers per section; the number of fibers varied from 0 to 4. In the pars intermedia the number of nerve fibers visible varied from none to 5 with an average of 2. Because of the anterior lobe injury the nerve fibers in this portion of the gland could not be counted.

Normal rats. In this species the majority of nerve fibers appeared to enter the gland as a bundle from the anterior part of the median eminence. A relatively small number of fibers was visible as a group in the posterior portion of the median eminence. After passing down the stalk these two large fiber trunks immediately broke up into smaller bundles which twisted tortuously in the infundibular process. At the periphery of the infundibular process the nerve fibers were scarce and it was possible in 6 specimens to trace many of them to a delicate pericellular network which enclosed single parenchymatous glandular cells described by Gersh (8). In one instance a nerve fiber could be seen which gave rise to three branches each of which immediately terminated around a different cell. In another specimen a nerve bundle was observed outside a capsular artery. Nerve fibers were fairly rare in the pars intermedia; 4 was the greatest number visible in a single 10 μ section. In preparations from 10 animals in which fibers could be seen they occurred as a rule once in every second section. They were found between the cells, chiefly at the periphery of the residual lumen of Rathke's pouch. Only one nerve fiber was observed extending between the infundibular process and the pars intermedia. Another was seen between the pars intermedia and the capsule. Nerve fibers were also scarce in the anterior lobe; in 8 glands an average of a single fiber occurred in each section. Only one nerve fiber was seen passing between pars intermedia and anterior lobe, and another between the latter and the capsule. Delicate pericellular baskets attached to nerve fibers were observed to surround some gland cells of the anterior lobe. These pericellular nets are similar to those which are seen around the parenchymatous glandular cells of the infundibular process. In 2 glands the filaments of the networks enclosing the gland cells were of the same thickness as the nerve fibers with which they were continuous.

Rats with superior cervical ganglionectomies. The nerve fiber distribution in these preparations was indistinguishable from that in normal glands. Pericellular networks could be seen around cells of the anterior lobe.

Stalk-cut rats. Only 2 preparations were useful for study. In the first rat which was operated on when 28 days old and killed 30 days later, the stalk was completely severed with only slight damage to the hypothalamus. In this gland no nerve fibers

were visible in any portion peripheral to the lesion. In the second rat which was operated on when approximately 5 weeks old and killed 6 weeks later, the stalk was incompletely sectioned; a thin strip of peripheral tissue on one side of the anterior part of the infundibular bulb and stem escaped injury. The lesion destroyed the whole of the posterior part of the median eminence and extended dorsally to include part of the hypothalamus directly above the median eminence. Nerve fibers were present only in the proximal part of the infundibular process, adjacent to its attachment with the stalk remnant. Unfortunately the pars intermedia and anterior lobe were not suitable for study.

DISCUSSION

Since the pyridine-silver method is so highly erratic in the success with which it impregnates nerve fibers, comparisons of the numbers of fibers in different sections of a single gland or in different glands are only suggestive. Possible confusion of nerve fibers with reticular or collagenous fibers was obviated by a fortunate accident whereby, in one series of glands, there was a thorough impregnation of non-nervous extracellular fibrils. The patterns which they formed were so different as to lead to a clear identification of nerve fibers. On the other hand there is some doubt concerning the interpretation of the pericellular fibrillar baskets in both anterior lobe and infundibular process. These terminal nets are thought by the authors to be nerve endings which are probably poorly preserved or poorly impregnated. Yet it is possible that they may represent artifacts caused by the use of ammoniacal alcohol, rightly considered to be a poor fixative. Whatever the nature or origin of these pericellular structures may be, they are seen only at the terminations of nerve fibers.

The character of the nerve fiber pattern of the hypophysis seems to be clearer in the rabbit than in the rat, though its appearance in both species is similar. In the rabbit the nerve fibers found in the hypophysial stalk are distributed chiefly to the infundibular process. Those in the periphery of the stalk are distributed principally to the proximal part of the infundibular process. Fibers from both parts of the neurohypophysis (infundibular process and stem) enter the pars intermedia which also receives a small number of sympathetic nerve fibers. Fibers from both parts of the neurohypophysis also reach the anterior lobe of the hypophysis. Those from the stalk enter directly, while those from the infundibular process pass through the pars intermedia around the edge of the residual lumen of Rathke's pouch. Fibers entering along capsular arteries constitute the sympathetic supply to the anterior lobe. In the rat, nerve fibers in the stalk pass into the infundibular process. There are indications that those in the periphery of the stalk are distributed to the proximal part of the process. Some nerve fibers terminate in what appears to be pericellular nerve nets which enclose single parenchymatous gland cells. Fibers of the pars intermedia are derived chiefly from those passing into it from the infundibular process. At least some of these nerve fibers extend into the anterior lobe which also may receive a few sympathetic fibers. Some nerve fibers in the anterior lobe appear to terminate as a net around gland cells. Fewer fibers are visible in the rat adenohypophysis than in that of the rabbit. This may be the result of differences in the size of the gland, or in the completeness of impregnation; or it may represent the actual state which obtains in these animals, since physiological evidence of neurally controlled anterior lobe functions is less convincing in the case of the rat. The close similarity of nerve fiber distribution in the adenohypophysis of the rabbit and cat (9) may well be significant since these species resemble each other in their post-coitus ovulatory reaction. It is felt that these observations furnish an anatomical basis for physiological evidence of a neural control of water exchange normally influenced by the neurohypophysis and of certain endocrine functions performed by the anterior lobe.

SUMMARY

The pyridine-silver method was used in a study of the hypophysis and adjacent hypothalamus of normal young rabbits and rats. Other animals were subjected to superior cervical ganglionectomy or to section of the hypophysial stalk and studied in the same way. Nerve fibers of the neurohypophysis originate in the hypothalamus. Most of these end in this part of the hypophysis but many continue into the pars intermedia and the anterior lobe. In the rabbit, and possibly also in the rat these fibers in the adenohypophysis are supplemented by a smaller number arising from the carotid plexus. In the rat, nerve fibers were seen to terminate in what have been interpreted as pericellular nerve nets around gland cells of the infundibular process and the anterior lobe.

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CORRELATION OF PHYSIOLOGICAL AND CYTOLOGICAL CHANGES IN THE NEUROHYPOPHYSIS OF RATS WITH EXPERIMENTAL DIABETES INSIPIDUS

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ANALYSES of experimental diabetes insipidus have been concerned only in a very limited way with the gland cells in the neurohypophysis which are believed to secrete the antidiuretic principle. It is true that Fisher, Ingram and Ranson (1) have described the total or partial disappearance of pituicytes which occur after interruption of the hypothalamico-hypophysial connections but the interpretation of the observations is admittedly vague, largely because of the non-specific nature of the staining methods employed. The rat is a more favorable animal for this type of study because the parenchymatous cells in the neurohypophysis of this animal are easily and precisely identified because their cytoplasm contains characteristic lipid droplets. The distribution of these cells in the infundibular process and stalk corresponds precisely with the sites from which the antidiuretic substance can be extracted (2). Moreover, it was found (2) that the gland cells hypertrophy when experimental evidence indicates that the store of active principle in the infundibular process is being depleted. Conversely, the gland cells become smaller when there is reason to believe that the antidiuretic substance is not being actively secreted. These induced cytological changes denote the state of activity of the cells and offer a convenient way to determine their behavior in various experimental conditions.

Experimental diabetes insipidus has been studied extensively in rats (3–6), but no report was made by any of these authors on the nature of the cell changes in the neurohypophysis. It was found that in some cases diabetes insipidus may develop gradually after a longer or shorter interval, reaching a maximum in 2 or 3 weeks. In others it may appear immediately, abruptly reaching a maximum and subsiding later to lower or normal levels; it then becomes more severe, attaining a peak in 2 or 3 weeks and maintains a high level thereafter. This 'permanent' phase differs from the preceding 'temporary' phase (when it occurs) in several respects which are listed in a recent review by Gersh (7). It is claimed (3, 6) that typical diabetes insipidus does not appear after removal of the anterior lobe or after complete hypophysectomy.

The present paper contains a description of changes observed in the parenchymatous cells of the neurohypophysis of the rat during the temporary and permanent phases of experimental diabetes insipidus and after hypophysectomy.[1] As controls, rats were used that had been subjected to the same operative procedure, but in which the lesion was such that they suffered no disturbance in their water intake and output. Attempts were made to correlate the nature of the lesion and the water intake and output with the cytology of the parenchymatous cells of the neurohypophysis.

For purposes of conciseness, arbitrary definitions of terms are employed in this paper. Rats are classified as 'permanently diabetic' if at the end of about 30 days fol-

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[1] The term "hypophysectomy" is employed to indicate the removal of the anterior lobe, pars intermedia and those parts of the neurohypophysis which are inferior to the diaphragma sellae turcicae.

lowing operation their urine output and water intake are at least 3 times as great as in the preoperative interval. Rats are called 'possibly diabetic' if at the end of one month following operation their water exchange is approximately two to three times that of the normal control period. Rats are said to have suffered from only 'temporary' diabetes if their water intake and output have returned to 'normal' at the end of one month after showing a transient increase as severe as that of permanent diabetes. Rats are called 'normal' whose urine output and water intake during the month following operation were never in excess of twice the values of the preoperative period. Some animals were borderline cases, but they have been placed arbitrarily in one or another category. A small number of animals were observed for shorter times. The anatomical terminology employed is that recommended by Rioch, Wislocki and O'Leary (8).

MATERIALS AND METHODS

This report is based on data obtained from 5 control animals which showed no diabetes after operation, 8 hypophysectomized rats, 8 diabetics, 3 possible diabetics and 5 rats that had temporary diabetes insipidus. In almost all cases the water exchange was followed through a control preoperative period. Food consisted of hard biscuits and weighed amounts of Purina pellets which had soaked up a known volume of water. Additional water was available from a measuring chamber devised by Richter (3). Food was changed daily and water replaced every second day or more frequently if necessary. Urine was collected in graduated cylinders from large funnels under the cages. Feces were screened out by a fine-meshed wire netting, interposed between funnel and cage. Readings of total water intake, food consumption and urine output were taken daily. All animals were weighed at intervals. Operations were carried out under nembutal injected intraperitoneally or under ether. Lesions were made mechanically through a temporal or a parapharyngeal approach or electrolytically by means of a unipolar electrode inserted with the aid of a simple stereotaxic instrument through a small trephined hole in the calvarium; in the latter case the large indifferent electrode was placed on the abdomen.

Animals were killed by opening the chest wall under ether anesthesia. After carefully dissecting away the base of the skull without injury to the dura, the hypophysis together with the adjacent hypothalamus was removed and fixed in Maximow's fluid. In many cases, the anterior lobe of the hypophysis was teased away and fixed separately in formalin-Zenker. The blocks were embedded in paraffin, and serial sections cut parasagittally at 5μ. Sections of the anterior lobe were stained with Mallory's triple stain or with hematoxylin and eosin; all other material was observed unstained. The tissues from one animal were fixed in formalin, sectioned as above, and stained with hematoxylin and eosin.

OBSERVATIONS

Normal control rats. In 2 animals lesions in the central nervous system were posterior to the hypophysis. In the other 3 the damage was in the region of the optic chiasm and in none was there any interference with the supraoptic nuclei, median eminence or hypophysial stalk. The water intake and output of these rats were followed for 11, 19, 21, 24 and 142 days following operation and showed no significant change from the preoperative level. The gland cells in the adeno- and neurohypophysis were quite normal in appearance.

Hypophysectomized rats. Five animals were subjected to complete hypophysectomy (*rat 117, 119, 120, 121, 124*). Water intake and urine output in 4 of these showed a sudden rise immediately after the operation but returned to normal in all cases within 10 days. Measurements were continued for 48 to 64 days following operation.

The fifth animal (120) failed to show this preliminary rise. At autopsy, hypophysectomy was found to be complete. In one preparation there was a small lesion just posterior to the border of the median eminence; in none of the others was there any further injury to the central nervous system. In all cases the parenchymatous cells in the remaining portion of the neurohypophysis fell within the normal range or slightly above it in size, number and distribution. Measurements of the diameters of the stalks showed them to extend over an average of 166 sections, as compared with 168 for the normal animals. Figure 1 shows the water intake and urine curve of one of the hypophysectomized animals (*rat 121*).

Hypophysectomy was less complete in 3 animals. In one (118) the only portion of the hypophysis remaining was the proximal tip of the infundibular process. There was a small lesion just posterior to the border of the median eminence. The water and

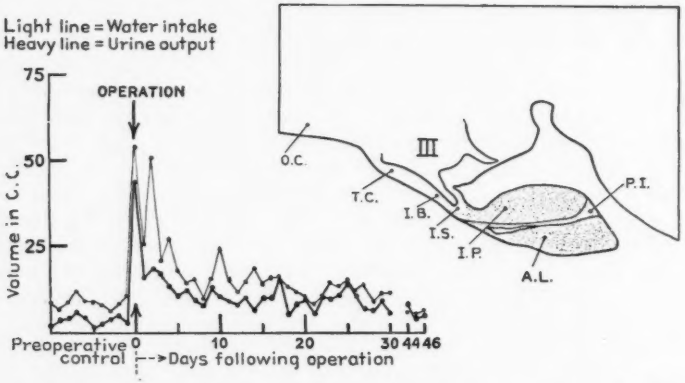

Fig. 1. WATER INTAKE AND URINE OUTPUT OF HYPOPHYSECTOMIZED *rat 121*, ♀; weight before operation 110 gm.; weight at autopsy 100 gm.; operation by parapharyngeal route. DIAGRAMATIC OUTLINE OF MID-SAGITTAL SECTION OF HYPOTHALAMIC REGION: A. L., anterior lobe; P.I., pars intermedia; I.P., infundibular process; I.S., infundibular stem; I.B., infundibular bulb; T.C., tuber cinereum; III, third ventricle.

urine curves were normal throughout the 61 days following operation except for a small drop during the first postoperative day. In another preparation (116) only the infundibular process and a part of the pars intermedia had been removed. A large part of the anterior lobe remained in good condition. There was some injury to the central nervous system posterior to the median eminence. The water curves were followed for 48 days. Both curves tended to be well above the preoperative values (bringing the animal almost in the class of possible diabetics). The cell population in the hypophysial stalk was normal, although the stalk itself was larger than usual. In the last preparation (79) almost the whole neurohypophysis was removed and about one-half of the anterior lobe. The lesion in the hypothalamus was enormous and spared perhaps only a small proximal part of the median eminence which seemed to contain a few small parenchymatous cells. Except for an initial postoperative rise there was only a moderate, variable rise in the water and urine curves to a slightly elevated new level.

Diabetic rats. Six rats were unquestionably diabetic; 2 others were to some extent atypical.

Rat 28. The water intake and urine output and other pertinent data are given in figure 2, with its accompanying legend. The stalk was almost completely severed at the base of the

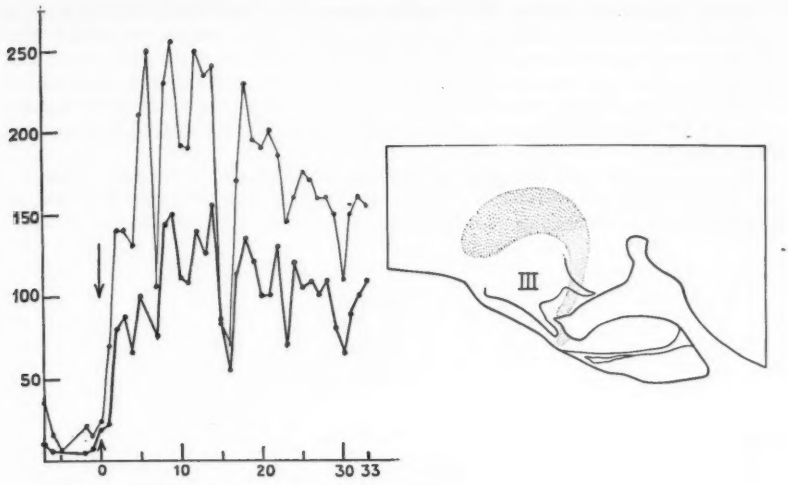

Fig. 2. Diabetic *rat 28*; ♂; weight before operation 194 gm.; weight at autopsy 197 gm.; operation by temporal route.

infundibular stem. In addition there was a unilateral region of necrosis extending somewhat beyond the midline which spread above the median eminence from behind, but spared this portion of the neurohypophysis. There were no differentiated cells in the distal two-thirds of the infundibular process, although a few scattered extracellular lipid droplets were found. Numerous large, hypertrophic cells were present in the proximal third of the process. The cell population in the stalk was the same as in the normal animals. The adenohypophysis appeared normal.

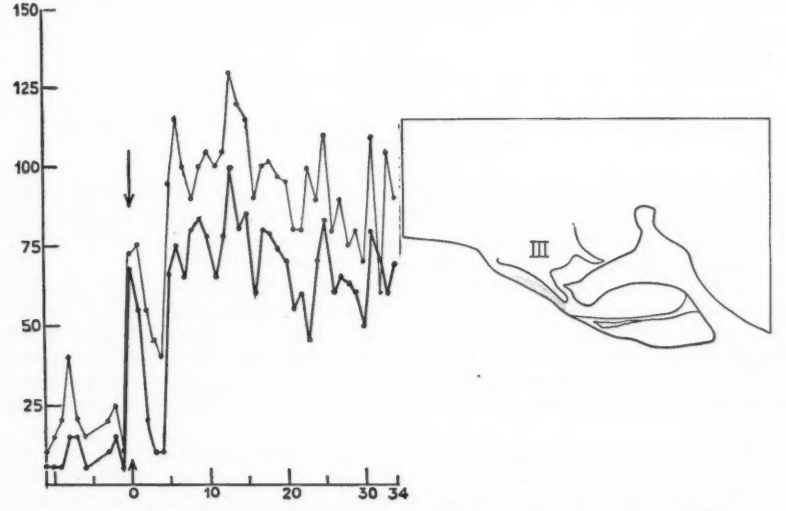

Fig. 3. Diabetic *rat 9*; ♂; weight before operation 108 gm.; weight at autopsy 186 gm.; operation by temporal route.

Rat 9. The anterior portions of the median eminence and infundibular bulb were almost completely destroyed, only a few scanty cells being visible in the proximal end of each. Normal cells were found in the posterior part of the median eminence, in the infundibular stem and in three small regions of the infundibular process. A few such cells were also found in the rest of the neurohypophysis where the cell population was composed chiefly of undifferentiated cells between which were scattered lipid droplets. The adenohypophysis appeared normal. (fig. 3).

Rat 101. A large necrotic region extending bilaterally anterior to the median eminence was occupied by numerous active phagocytes, which were also present in the thickened underlying dura mater. In addition, there appeared to be a small injury at the base of the infun-

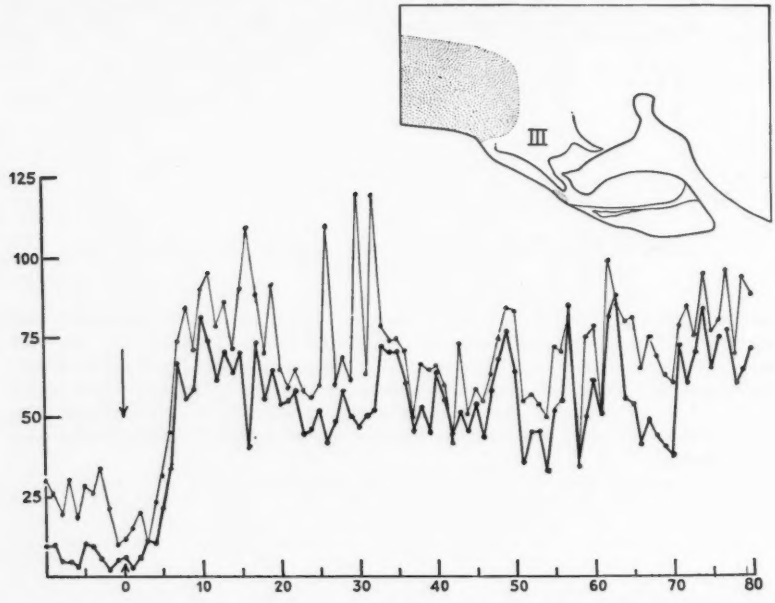

Fig. 4. Diabetic *rat* 101; ♂; weight before operation 290 gm.; weight at autopsy 380 gm.; operation by parapharyngeal route.

dibular bulb. The distal half of the infundibular process contained few differentiated cells. These as well as other cells in the uninjured parts of the stalk were markedly hypertrophic. Except that the residual lumen of Rathke's pouch was filled with a stainable material containing phagocytes, the adenohypophysis appeared normal. (fig. 4).

Rat 69. The animal was found dead; its brain was fixed by immersion in formalin. The connections between infundibular process and bulb had been completely severed. The anterior portion of the stalk was extremely thin. A bilateral lesion beginning anterior to the median eminence extended dorsally into the hypothalamus. The infundibular process was smaller than usual and seemed necrotic. The pars intermedia appeared to be uninjured. About one-third of the anterior lobe was replaced by scar tissue or contained agranular cells. (fig. 5).

Rat 92. Just below the infundibular bulb the stalk was completely severed, except for a strip 75μ wide at the periphery on one side. In addition there was a narrow mid-line lesion extending superiorly from behind the median eminence; in the first operation the right optic nerve had been cut. The thickness of the remaining portion of the stalk extended through 280 sections. The parenchymatous cells in the stalk proximal to the lesion were larger and

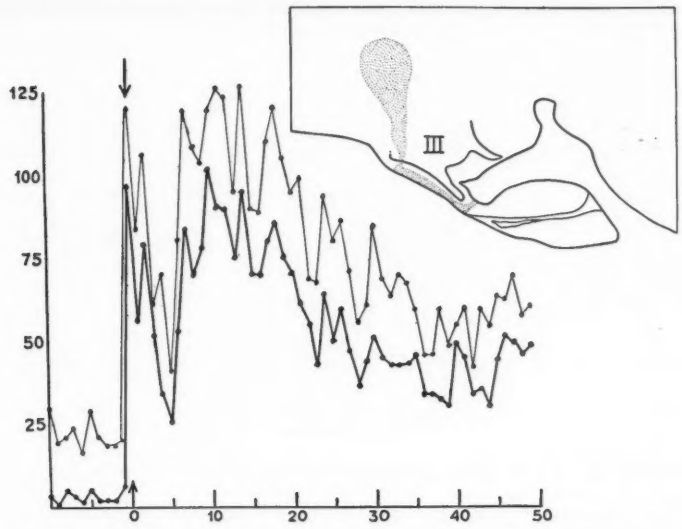

Fig. 5. Diabetic *rat* 69; ♀; weight before operation 175 gm.; weight at autopsy 168 gm.; operation by parapharyngeal route. Food intake during first 5 days following operation greatly reduced; food consumption not decreased in all other experimental rats.

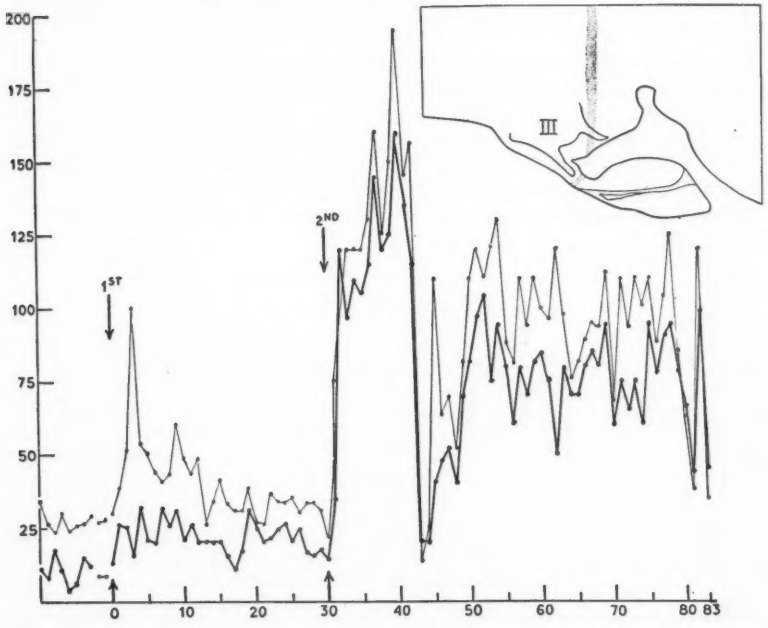

Fig. 6. Diabetic *rat* 92; ♂; weight before first operation 245 gm.; weight before second operation 444 gm.; weight at autopsy 245 gm.; ½ cc. obstetrical pituitrin (5 U) injected 43 days after first operation; electrolytic lesions.

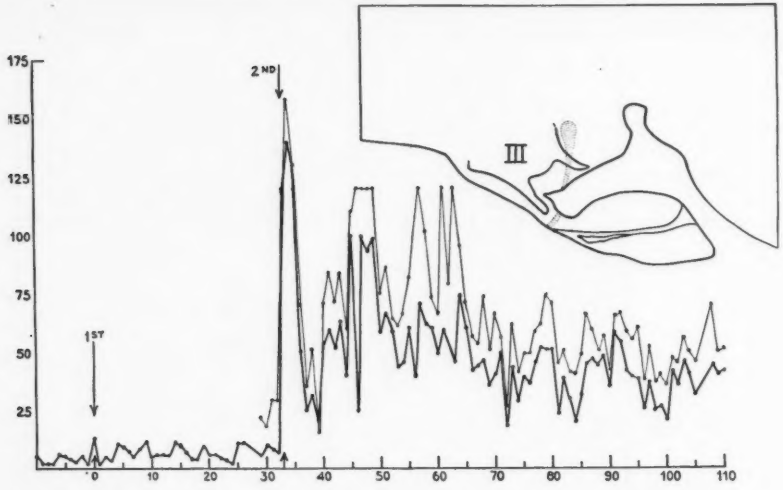

Fig. 7. Diabetic *rat 94*; ♀ ; weight before first operation 200 gm.; weight before second operation 190 gm.; weight at autopsy 245 gm.; electrolytic lesions.

more numerous than in normal animals. In more than half of the infundibular process the cells were atrophic and numerous lipid droplets could be seen scattered between them. In the remaining portion a few normal-looking differentiated cells were distributed among the numerous atrophic cells. The anterior lobe was displaced but appeared cytologically normal. (fig. 6).

Rat 94. The stalk was completely severed between the infundibular process and stem. There was also a narrow necrotic region extending dorsally from behind the median eminence. The differentiated cells in the proximal portion of the neurohypophysis were very large and quite numerous; the whole stalk was larger than usual. The infundibular process was very small and contained a few small cells in whose cytoplasm were small numbers of tiny lipid droplets. Numerous apparently extracellular lipid droplets scattered between atrophic cells were a prominent feature of the neural lobe. The adenohypophysis appeared normal. (fig. 7).

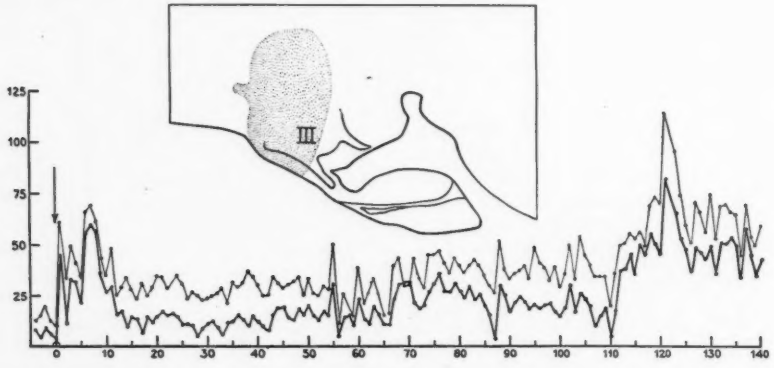

Fig. 8. Diabetic *rat 55*; ♀ ; weight before operation 166 gm.; weight at autopsy 251 gm.; electrolytic lesion.

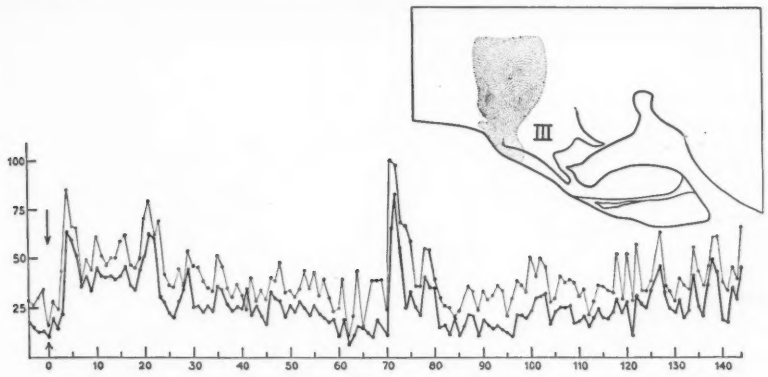

Fig. 9. Diabetic *rat 144*; ♀; weight before operation 198 gm.; weight at autopsy 230 gm.; electrolytic lesion.

Rat 55. The lesion was large, destroying the tissue on one side of the anterior portion of the median eminence and extending beyond the mid-line to spare only about the peripheral one-third of the median eminence of the other side. Most of the hypertrophic cells in the remnant of the stalk were peculiar in that the normally discrete lipid cytoplasmic inclusions had coalesced to form single droplets or several irregularly large globules which occupied almost the whole cytoplasm. There were also numerous cells intermediate between these and normal ones. In addition, lipid droplets were observed apparently scattered in the intracellular spaces. The adenohypophysis appeared normal. (fig. 8).

Rat 144. The lesion extended bilaterally just anterior to the median eminence sparing only a thin strip of the ventral portion of the hypothalamus. Abnormal cells similar to those found in *rat 55* were present throughout the whole neurohypophysis. Extracellular debris was especially prominent in the infundibular stem and bulb, but was also present in the neural process. The adenohypophysis appeared normal. (fig. 9).

Rats with possible diabetes. *Rat 44*. The left optic nerve was severed. There was a large lesion in the mid-line anterior to the optic chiasm and a very small one in the posterior por-

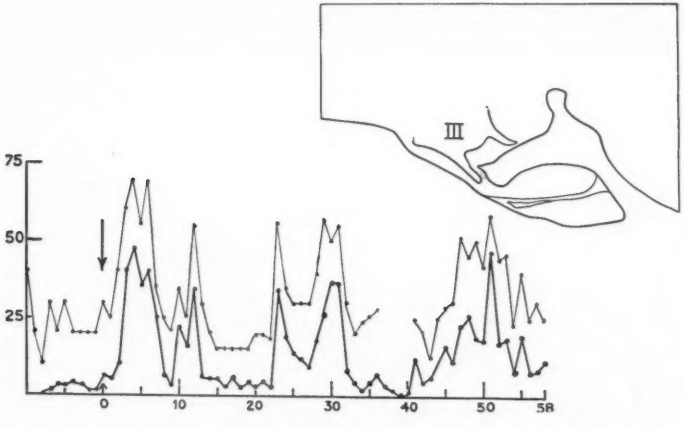

Fig. 10. *Rat 44* with possible diabetes; ♂; weight before operation 127 gm.; weight at autopsy 160 gm.; operation by temporal route.

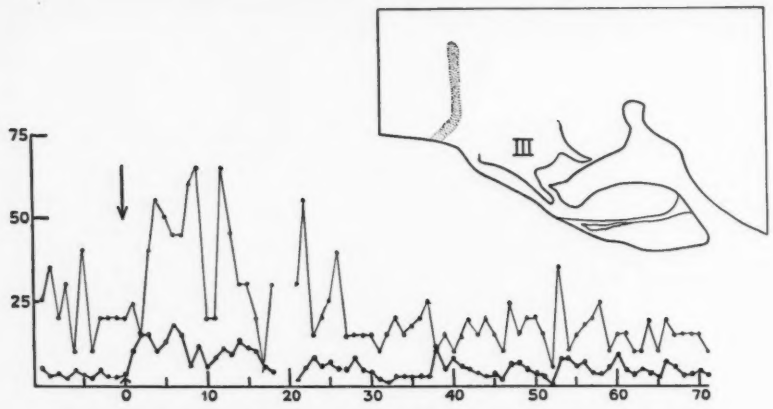

Fig. 11. *Rat* 24 with possible diabetes; ♀; weight before operation 180 gm.; weight at autopsy 185 gm.; operation by temporal route.

tion of the infundibular bulb. The cells in the adeno- and neurohypophysis appeared normal. (fig. 10).

Rat 24. This animal might be classified as suffering from temporary diabetes. The lesion was situated just behind the level of the optic chiasm; it was chiefly unilateral and extended very slightly beyond the midline. The parenchymatous cells in the neurohypophysis were markedly hypertrophic. The adenohypophysis appeared normal. (fig. 11).

Rat 52. The lesion was confined to one side. It began directly behind the level of the optic chiasm and extended posteriorly. The differentiated cells in the stalk were normal in appearance; those in the infundibular process were larger and more numerous than usual. The adenohypophysis seemed normal. (fig. 12).

Rats with temporary diabetes. Rat 1. A small unilateral lesion in the optic tract was the only detectable injury. The differentiated paenchymatous cells in all parts of the neurohypophysis were larger and more numerous than normal. In some cells lipid droplets had coalesced. A small necrotic region was found in the anterior lobe where the eosinophils were smaller than normal and the proportion of non-granular cells appeared to be excessive. The pars intermedia seemed normal. (fig. 13).

Rat 4. The chief lesion was unilateral and anterior to the median eminence. A few scattered wandering cells were found above the optic chiasm. The cells of the neurohypophysis

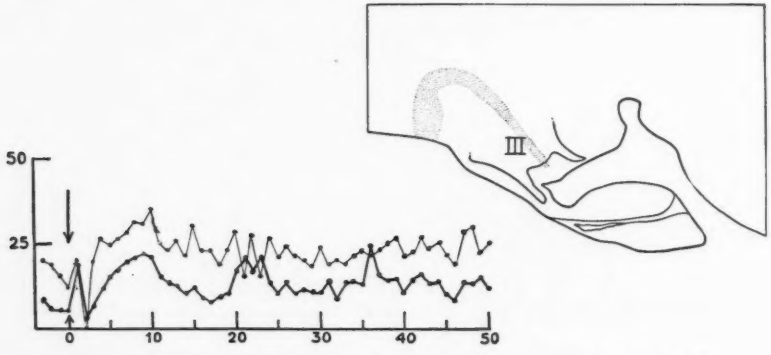

Fig. 12. *Rat* 52 with possible diabetes; ♀; weight before operation 170 gm.; weight at autopsy 208 gm.; electrolytic lesion.

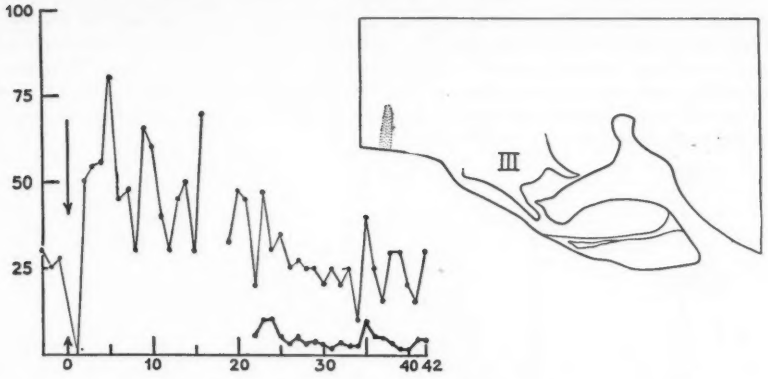

Fig. 13. *Rat 1* with temporary diabetes; ♀; weight before operation 120 gm.; weight at autopsy 165 gm.; operation by temporal route.

were to some extent more numerous and larger than normal. The adenohypophysis appeared normal. (fig. 14).

Rat 56. The lesion in the brain was lateral to the tuber cinereum and the left optic nerve had been cut. The differentiated cells in the infundibular process were larger and more numerous than normal, while those in the remainder of the neurohypophysis fell within the normal range. The adenohypophysis appeared normal. (fig. 15).

Rat 5. There was a small unilateral injury to the optic chiasm and another extensive one on the same side which lay superior to and spared the neurohypophysis. The cells in the infundibular process appeared normal, while those in the remainder of the neurohypophysis were hypertrophic. A few cells with coalesced droplets could be found in all parts. The adenohypophysis seemed normal. Following operation the water intake fell slightly and remained low for 6 days. During the next 3 days the water intake rose to 5 times that of the normal preoperative period. It then fell gradually and reached the normal level on the twelfth day after operation at which time the animal was autopsied.

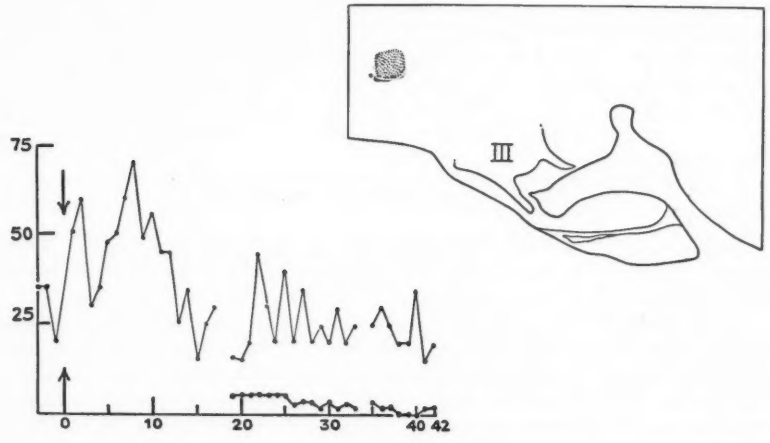

Fig. 14. *Rat 4* with temporary diabetes; ♀; weight before operation 90 gm.; weight at autopsy 136 gm.; operation by temporal route.

Rat 23. The lesion was unilateral and extended above the median eminence on that side. There was no injury on the other side. The differentiated cells in the infundibular bulb and median eminence appeared normal. In the remainder of the neurohypophysis there were numerous hypertrophic cells in many of which the lipid droplets had coalesced. In addition, extracellular lipid droplets were present in the infundibular process. The adenohypophysis seemed normal. (fig. 16).

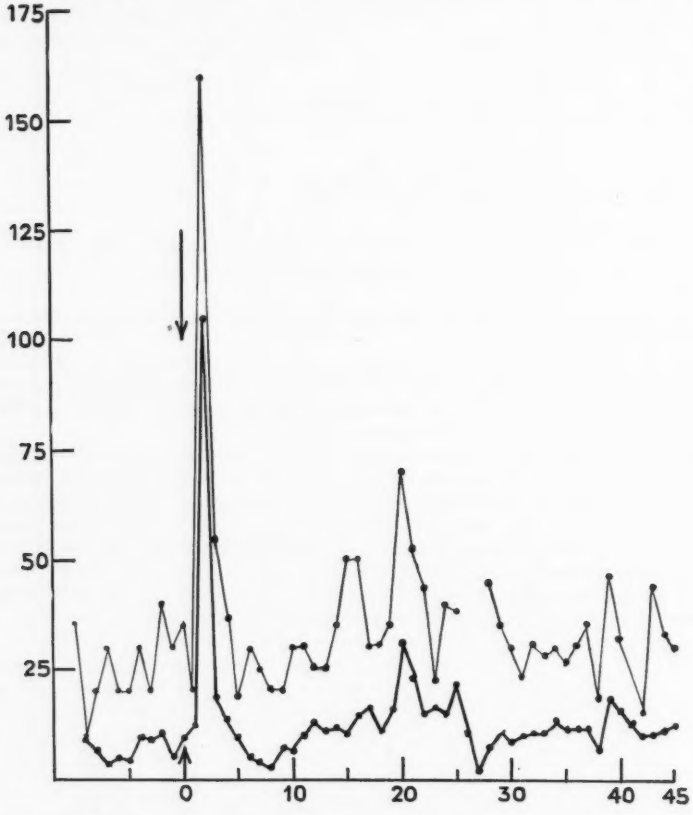

Fig. 15. *Rat* 56 with temporary diabetes; ♂; weight before operation 310 gm.; weight at autopsy 305 gm.; operation by temporal route.

Summary. In control rats, the lesions were in the brain stem posterior to the median eminence or anterior to the optic chiasm; the cells in the neurohypophysis were well within the normal range in respect to size, number and distribution.

After complete, uncomplicated hypophysectomy the intake and output of water showed a rapid rise but shortly returned to a normal level. The parenchymatous cells in the stalk of such animals showed either no change from normal or a tendency to hypertrophy. Similar phenomena were observed when a small portion of the neurohypophysis remained attached to the stalk. One case of partial hypophysectomy in which a large part of the anterior lobe remained and which was complicated by extensive brain lesions above the median eminence showed a temporarily disturbed water exchange. Another similar preparation in which the brain lesion was in the region of the

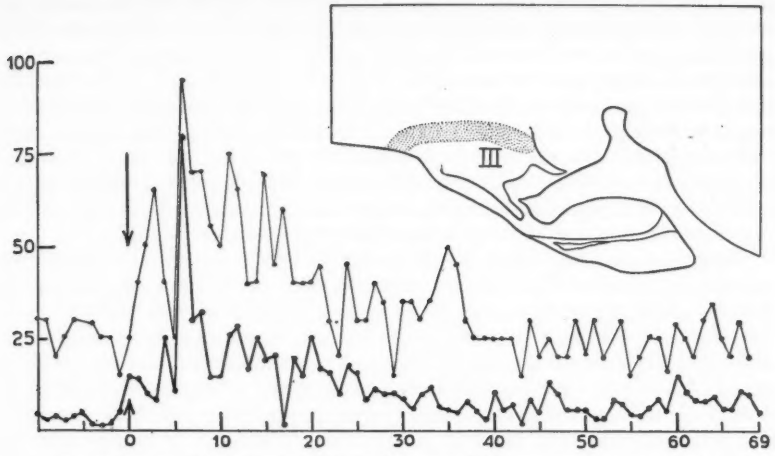

Fig. 16. *Rat* 23 with temporary diabetes; ♀; weight before operation 213 gm.; weight at autopsy 321 gm.; operation by temporal route.

mammillary bodies also showed a temporary polyuria and polydipsia. In general, following hypophysectomy a mild cellular hypertrophy appeared to take place in the stalk which, however, was not increased appreciably in size.

Four types of lesions were observed in the permanently diabetic animals. (a) In rats 92 and 94, stalk section had evidently produced a marked cellular hypertrophy with an appreciable increase in the size of the portion of the stem proximal to the lesions and a noticeable atrophy of the parenchymatous cells of the infundibular process. (*b*) In *rat* 69, complete stalk section with injury to the anterior portion of the hypophysial stalk reduced the size of the neurohypophysis markedly. (*c*) In rats 9, 28, and 101, in which the hypophysial stalk was greatly injured, cellular atrophy in some regions of the neurohypophysis was compensated by marked hypertrophy of remaining cells. (*d*) Rats 55 and 144, were atypical since diabetes insipidus reappeared a considerable time after the subsidence of an initial rise in the water intake and output. In both of these animals a large brain lesion anterior to the median eminence destroyed a great part of the median eminence. The remainder of the neurohypophysis was characterized by the presence of much cellular debris and of parenchymatous cells whose lipid inclusions had coalesced. In all cases but one there were large brain lesions.

In rats 24, 44, and 52, which suffered from a possible diabetes insipidus, extensive unilateral or bilateral lesions in the region of the optic chiasm had been made with little or no direct injury to the neurohypophysis. In two preparations cellular hypertrophy was apparent, but no cellular reactions were visible in the third specimen. There was no apparent increase in the size of the stalk.

Four rats that had suffered from temporary diabetes showed lesions in the region of the optic chiasm. Cellular hypertrophy was detectable in the neurohypophyses of all. A fifth rat (*rat* 5) which had a large lesion superior to the median eminence did not show such a response and no apparent increase in the size of the stalk was detected.

The adenohypophysis of all types of animals having functional disturbances showed no abnormalities of any significance.

DISCUSSION

Interpretation of the observations recorded in this paper are based on certain primary data. (*a*) In an effort to prevent urinary loss of water, parenchymatous cells in

the neurohypophysis hypertrophy when an increased amount of antidiuretic substance is being secreted (2). During this period, the total content of the active principle in the gland is being decreased (2, 9, 10). (*b*) The chief innervation of the neurohypophysis is derived from the supraoptic nuclei (see Rasmussen, 11). The fibers are distributed throughout the gland and terminate in what are believed to be nerve endings around the parenchymatous cells (12). The chief assumption made (supported by some data) is that a process analogous to paralytic secretion occurs in the neurohypophysis when its cells are denervated. On the basis of these facts and assumptions it is possible to offer a simple interpretation of temporary and permanent diabetes insipidus in rats, and of the effects of complete hypophysectomy on water balance.

In general, temporary diabetes follows lesions which permanently injure a considerable part but not all of the supraoptico-hypophysial system. Small or equally large lesions outside of this system have no noticeable effect on water exchange or on the gland cells of the neurohypophysis. One case of temporary diabetes was complicated by an extensive brain lesion which may have so altered visceral metabolic functions as to result in a damping of what might have been otherwise a full-blown permanent disturbance in the water balance. In the rats suffering only from the uncomplicated temporary stage, interference with nerve fibers may have inactivated an equivalent number of parenchymatous cells. Under these conditions, the cells in the neurohypophysis which still have an intact nerve supply would show a compensatory hypertrophy. Such a 'corrective' reaction has been observed in other endocrine glands such as pancreas, adrenal cortex and thyroid when a large portion of glandular tissue is inactivated (i.e., removed).

After 'complete' hypophysectomy a similar but shorter increase in water exchange takes place. The initial rise may be attributed in this case to the loss of numerous cells which were actively engaged in secreting an antidiuretic substance. The water and urine curves rapidly return to normal levels because of two possible factors: an increase in the secretory activity of the parenchymatous cells resident in the remaining portion of the stalk, and a deranged metabolism referable to general endocrine imbalance in the body following removal of the anterior lobe of the hypophysis. The first possibility is supported by the observation that the remaining parenchymatous cells are usually hypertrophied in hypophysectomized rats. The argument is strengthened also by the finding (13) that the amount of antidiuretic substance in the tuber cinereum is raised in hypophysectomized dogs as compared with normals. A discussion of the second possibility is given in a review by Gersh (7).

In all our diabetic animals the lesions destroyed the greatest part of the innervation of the neurohypophysis, either by complete stalk section, destruction of the chief part of the stalk or serious injury to nervous tissue between the median eminence and the supraoptic nuclei. This denervation of a large number of parenchymatous cells was followed by their atrophy. Cellular degeneration may have followed also from prolonged compensatory hypertrophic activity in the still innervated regions (especially in rats *55* and *144*). Indeed, in these 2 animals, the delayed frank diabetic stage may have been reached long after the operation only because the cells which hypertrophied had finally reached the stage of exhaustion atrophy.

It should be pointed out that some cells which appear normal may occur even in a completely denervated region of the neurohypophysis. This may be correlated with the fact that the posterior lobe of the diabetic cat always shows the presence of some antidiuretic activity (1). The occurrence of such cells in regions deprived of nerve fibers is paralleled in other glands by similar phenomena. The invariable occurrence of such cells over a long time suggests that in continuous paralytic secretion persistently active cells are being derived constantly from less active and less differentiated cells.

Continuous hyperactivity in these cells may then result in exhaustion atrophy. This is reflected by the presence of degenerating cells visible in preparations long after operation. In some of our fully diabetic animals the urine flow tended to decrease. This was observed previously in rats (4, 6) and in other animals (1). This compensatory response is evidence that factors other than the antidiuretic substance play a role in water metabolism, at least under certain conditions.

Briefly stated, the theory which seems to explain best the observations on experimental diabetes insipidus in the rat is the following. The parenchymatous gland cells of the neurohypophysis are released from nervous control when the nerve fibers which innervate them are destroyed. In this state they atrophy. As part of the picture of paralytic secretion the less differentiated cells also denervated may then hypertrophy and subsequently atrophy through exhaustion. When the number of inactive or atrophic cells is large there is an attempt at compensatory hypertrophy. If this is successful and the remaining cells can secrete enough antidiuretic substance, the animal will show only the temporary phase of diabetes insipidus. If the hypertrophic cells are too few in number or the hypertrophy is not marked enough and the antidiuretic principle is secreted below the physiological level, the animal will suffer from a persistent, mild excess in urine output and water intake. If the differentiated cells are too few in number and the hypertrophic cells secrete to exhaustion permanent diabetes will ensue. The initial state of water imbalance in 'complete' hypophysectomy is the result of removing a major source of antidiuretic substance. Water balance will be restored when this deficiency is compensated for by cellular hypertrophy. Later, when removal of the anterior lobe has produced an endocrine imbalance in the body this cellular reaction may become unnecessary and the parenchymatous cells may revert to their normal physiological state and appearance. The theory as proposed agrees with and extends the main tenets of the neuro-hormonal theory of the causation of diabetes insipidus sponsored by the Ranson school.

SUMMARY

Water intake and urine output were determined in rats with various types of hypothalamic lesions. The animals were classified as operated normal controls, rats with temporary diabetes insipidus, possibly diabetic rats, permanently diabetic rats, and hypophysectomized rats. The parenchymatous glandular cells of the neurohypophysis were described, and deviations from the normal state which occur in these animals were pointed out. The cellular reaction in the neurohypophysis was correlated with changes in water intake and urine output. A theory of the role of the gland cells in water balance was proposed that agrees with and extends the main tenets of the neurohormonal theory of the causation of diabetes insipidus sponsored by the Ranson school.

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SUCCESSFUL RESPONSES IN DIABETES MELLITUS OF THE MENOPAUSE PRODUCED BY THE ANTAGONISTIC ACTION OF SEX HORMONES ON PITUITARY ACTIVITY

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RECENT STUDIES have demonstrated the importance of the hypophysis in the hormonal factors which are responsible for the onset of diabetes mellitus, especially when the first symptoms appear after the menopause. Since Houssay and his collaborators (1) have presented convincing evidence that the diabetogenic principle of the anterior pituitary seems to be the prime mover in the hormonal dysfunction which produces diabetes, we tried to obtain the inhibition of this diabetogenic action by the administration of sex hormones in large doses. In order to test our working hypothesis, 40 patients were treated with an absolutely free diet, without any restrictions and without insulin. The constant improvement observed in all the cases, as measured by the dextrose tolerance test, is shown in the accompanying table.

DISCUSSION

There is clinical and experimental evidence that pituitary activity may be inhibited by the administration of the sex hormones. Zondek (2) and Allen (3) have demonstrated that anterior pituitary functions, in particular of the gonadotropic and growth principles, may be suppressed by injection of large doses of estrogenic substances.

We have reported (4) a case of pregnancy in which the Friedman reaction was inhibited by the female sex hormone (estradiol benzoate); after 5 weekly injections of 50,000 R.U. (5 mg.), the urine injected into infantile rabbits did not produce any ripe follicles or hemorrhage. Lately, a patient with acromegaly has been described by Stephens (5) in whom persistent milk secretion from both breasts was suppressed by estrogenic therapy. Nelson (6) has demonstrated that in the experimental animal previously treated with estrone, the lactation ordinarily induced by the injection of pituitary extracts fails to appear.

The literature contains much convincing evidence that the sex hormones inhibit pituitary activity, but none of the investigators has considered in particular the inhibition of the diabetogenic principle. Indeed, Rathery and Froment (7) have described an "hypoglycemic action" of testosterone propionate; in such a case, it could be justifiably assumed that the inhibition of the diabetogenic principle is responsible for the hypoglycemia. It seems also justified to postulate that this inhibition can be exerted in the same way on the ketogenic principle produced by the anterior pituitary.

It is not possible at present to decide whether the inhibition is only due to the action of estrin, or whether it is induced by both hormones, estrin and progesterone. In fact, Laroche, Simmonet and Bompard (12) have demonstrated that progesterone,

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Case No., age, yr.	Menses[1]	Treatment		Dextrose tolerance test. Blood sugar		
		Estrin	Progesterone	Hour	Before treatment, mg. %	After treatment, mg. %
1, 56	11/28/4/47	5 mg. 3×wk., 250 mg.	100 mg. 2×wk., 500 mg.	0	155	100
				1	205	125
				2	145	97
2, 54[2]	12/22/6/50	5 mg. 3×wk., 100 mg.	10 mg. 2×wk., 200 mg.	0	162	85
				1	300	180
				2	155	105
3, 49[3]	?/24/7/44	10 mg. 3×wk., 520 mg.	10 mg. 3×wk., 520 mg.	0	200	122
				1	400	168
				2	200	115
4, 52[2]	11/22/8/49	10 mg. 3×wk., 1200 mg.	5 mg. 3×wk., 600 mg.	0	172	88
				1	295	130
				2	144	100
5, 61[2]	13/30/5/50	5 mg. 3×wk., 500 mg.	5 mg. 3×wk., 500 mg.	0	165	105
				1	395	145
				2	180	112
6, 58	12/30/?/44	5 mg. 3×wk., 215 mg.	2 mg. 3×wk., 82 mg.	0	152	88
				1	190	97
				2	166	75
7, 62	?/23/6 to 9/49	10 mg. 2×wk., 180 mg.	5 mg. 2×wk., 90 mg.	0	172	112
				1	285	112
				2	—	95
8, 49	12/28/4/46	5 mg. 3×wk., 220 mg.	2 mg. 3×wk., 88 mg.	0	145	94
				1	145	125
				2	105	92
9, 47	11/29/5/39	5 mg. 3×wk., 240 mg.	1 mg. 3×wk., 48 mg.	0	66	105
				1	310	215
				2	200	165
10, 49	12/?/?/46	5 mg. 3×wk., 155 mg.	2 mg. 3×wk., 62 mg.	0	157	75
				1	222	155
				2	165	92
11, 52[3]	11/20/10/47	15 mg. 3×wk., 1080 mg.	10 mg. 3×wk., 720 mg.	0	255	117
				1	420	200
				2	300	99
12, 54[2]	11/20 to 25/5/47	5 mg. 4×wk., 740 mg.	5 mg. 4×wk., 740 mg.	0	195	108
				1	350	145
				2	160	90
13, 49	12/27/7/45	5 mg. 3×wk., 305 mg.	2 mg. 3×wk., 122 mg.	0	155	95
				1	270	140
				2	165	105
14, 52	13/30/4/50	5 mg. 3×wk., 260 mg.	5 mg. 3×wk., 260 mg.	0	160	115
				1	160	115
				2	145	100

[1] Figures refer, respectively, to: age in years at onset; usual interval, in days, between menses usual duration, in days, of flow; age, in years, at menopause.

[2] Glycosuria present.

[3] Glycosuria and ketonuria present.

Case No., age, yr.	Menses[1]	Treatment		Dextrose tolerance test. Blood sugar		
		Estrin	Progesterone	Hour	Before treatment, mg. %	After treatment, mg. %
15, 49[2]	10/15 to 20/8/43	15 mg. 3×wk., 1155 mg.	10 mg. 3×wk., 770 mg.	0	300	140
				1	485	227
				2	325	150
16, 61[2]	12/?/?/48	10 mg. 3×wk., 920 mg.	5 mg. 3×wk., 460 mg.	0	290	122
				1	475	177
				2	305	112
17, 49[2]	11/28/7/44	5 mg. 3×wk., 210 mg.	2 mg. 3×wk., 84 mg.	0	198	92
				1	375	100
				2	210	85
18, 50	10/24/8/49	5 mg. 3×wk., 255 mg.	2 mg. 3×wk., 102 mg.	0	166	100
				1	270	133
				2	165	98
19, 47[2]	?/28/6/41	10 mg. 2×wk., 480 mg.	5 mg. 2×wk., 240 mg.	0	192	107
				1	335	147
				2	285	110
20, 53	11/20/8/49	5 mg. 3×wk., 180 mg.	2 mg. 3×wk., 72 mg.	0	160	—
				1	247	185
				2	145	120
21, 49[2]	10/15/ to 20/9/43	5 mg. 4×wk., 180 mg.	2 mg. 3×wk., 72 mg.	0	75	125
				1	425	200
				2	395	130
22, 57	12/28/5/47	5 mg. 3×wk., 260 mg.	5 mg. 3×wk., 260 mg.	0	157	76
				1	205	125
				2	145	80
23, 50	14/31/3/46	5 mg. 3×wk., 420 mg.	2 mg. 3×wk., 168 mg.	0	145	99
				1	240	155
				2	157	100
24, 48	11/27/6/46	5 mg. 3×wk., 190 mg.	2 mg. 3×wk., 76 mg.	0	175	125
				1	300	190
				2	220	135
25, 52	15/30 to 45/4/44	5 mg. 2×wk., 160 mg.	2 mg. 2×wk., 64 mg.	0	155	110
				1	190	145
				2	100	105
26, 49	12/24/7/42	5 mg. 3×wk., 210 mg.	2 mg. 3×wk., 84 mg.	0	148	100
				1	277	170
				2	155	97
27, 53	11/27/5/48	5 mg. 3×wk., 205 mg.	5 mg. 2×wk., 140 mg.	0	166	112
				1	195	100
				2	160	88
28, 53[2]	10/?/9/47	10 mg. 4×wk., 1280 mg.	5 mg. 4×wk., 640 mg.	0	340	180
				1	455	285
				2	400	285
29, 52[2]	?/25 to 35/?/43	5 mg. 4×wk., 325 mg.	2 mg. 4×wk., 130 mg.	0	205	122
				1	276	190
				2	205	111

Case No., age, yr.	Menses[1]	Treatment		Dextrose tolerance test. Blood sugar		
		Estrin	Progesterone	Hour	Before treatment, mg. %	After treatment, mg. %
30, 49	12/30/2/39	5 mg. 3×wk., 420 mg.	5 mg. 3×wk., 420 mg.	0 1 2	185 377 167	105 250 140
31, 54	11/30/5 to 10/48	5 mg. 3×wk., 260 mg.	2 mg. 3×wk., 104 mg.	0 1 2	170 222 188	112 165 110
32, 50	13/32/4/40	5 mg. 3×wk., 270 mg.	1 mg. 3×wk., 54 mg.	0 1 2	152 199 150	82 145 90
33, 47[2]	?/26/7/45	5 mg. 3×wk., 180 mg.	2 mg. 3×wk., 72 mg.	0 1 2	190 305 200	100 205 110
34, 49	12/28/6/44	10 mg. 2×wk., 370 mg.	5 mg. 2×wk., 185 mg.	0 1 2	176 240 168	105 165 112
35, 50	?/25/7 to 15/45	5 mg. 3×wk., 160 mg.	2 mg. 3×wk., 64 mg.	0 1 2	145 145 140	125 122 130
36, 52	12/30/6/47	5 mg. 3×wk., 215 mg.	2 mg. 3×wk., 86 mg.	0 1 2	185 345 205	110 145 100
37, 54[2]	?/25/?/45	10 mg. 3×wk., 430 mg.	5 mg. 1×wk., 14 mg.	0 1 2	255 370 264	117 152 115
38, 49	11/24/7/44	5 mg. 3×wk., 255 mg.	2 mg. 3×wk., 102 mg.	0 1 2	185 257 177	96 134 75
39, 51[2]	12/?/?/43	5 mg. 3×wk., 310 mg.	2 mg. 3×wk., 124 mg.	0 1 2	200 375 217	110 184 145
40, 51	11/26/6 to 10/43	5 mg. 3×wk., 325 mg.	2 mg. 3×wk., 130 mg.	0 1 2	160 235 152	100 144 110

given subcutaneously, was effective in reducing the output of gonadotropic hormone in urine and relieving symptoms of the menopause in several cases. These data suggest that both principles, estrin and progesterone, are responsible for the inhibition of the pituitary activity. It is as if nature, in its economy, stops somatic growth by increasing sex hormone concentration (13), when the body needs no further growth. A dysfunction in this delicate hormonal balance becomes the primum movens of diabetes in the menopause when sex hormone concentration decreases.

SUMMARY

A group of forty women in the menopause or the postmenopause presenting diabetes mellitus, formed the material for this study. All of the patients treated

showed evidence of a disturbed hormonal balance characterized by hyperactivity of the pituitary, which should be attributed to the diminution of the gonadal activity. Successful responses in 100% of the reported patients, including some severe cases of diabetes with glycosuria and ketonuria, emphasize the necessity of separating this special type of climateric diabetes characterized in particular by a diminished carbohydrate tolerance, which differs in no essential from that of Cushing's disease.

While insulin treatment and diet were carried out in our patients without any other endocrine therapy, the disturbed carbohydrate metabolism remained almost unchanged. Successful responses were obtained only with estrin and progesterone in large doses. The condition of the patients after stopping treatment, showed the remarkable effects of this therapy. No implication is intended that our patients were genuinely cured. However, the marked inhibition exerted on the pituitary activity by the sex hormones makes this therapy of much interest.

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INHERITANCE OF GLUCOSE TOLERANCE[1]

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RECOGNIZING the evidence for the familial transmission of diabetes, reviewed by White and Pincus (1) and for a low glucose tolerance as the usual precursor of clinical diabetes and the earliest dependable prognostic sign, it seems that the pattern for the inheritance of diabetic traits could be most satisfactorily determined by a study of the inheritance of glucose tolerance. This approach has been used by Pincus and White (2) in man and a similar investigation has been made by the present authors in rats.

For several years, the authors (3, 4, 5) and subsequently others (6, 7) have been studying in a strain of rats certain diabetic characteristics: (*a*) high fasting blood sugar, (*b*) low glucose tolerance, (*c*) low R. Q. after glucose, (*d*) exaggerated response to epinephrin and (*e*) obesity. A hyperactivity of the anterior pituitary has been postulated as offering the most satisfactory common denominator for these and the other abnormalities of this strain (8). Such a strain would appear to provide an acceptable tool for a study, under rigidly controlled conditions, of the familial transmission of a low glucose tolerance of pituitary origin, and such an investigation conceivably may amplify the present conception of the inheritance of human diabetes. It must be emphasized, however, that no member of this colony has developed spontaneously a condition analogous to diabetes mellitus, and the authors have been unable to find the provocative force required to produce this transformation.

EXPERIMENTAL METHODS

Glucose tolerance tests. These tests followed the procedure previously reported (4) except that the blood sugar determinations were made only ½ and 5 hours subsequent to the administration of the glucose. The criteria for normal tests, the same as those originally adopted (4, 5) and subsequently used throughout this series of studies, demand that, (*a*), the ½-hour blood sugar must be less than 300 mg. %; (*b*), the 5-hour value must be less than 180%; (*c*), the fall for this interval must be at least 40 mg.% for values of 200 mg.% or less at the half-hour, or 60 mg.% for values over 200 at the half-hour. The determinations were made when the males were 100 days of age or over and the females 140 days or older. Our data have shown that after these ages the increase in the number of animals giving diabetic-like curves is unimportant (5).

Rat stock. Approximately two-thirds of the males and one-half of the females from our Yale (Y) stock gave diabetic-like, low tolerance curves. Attempts to produce a family of rats showing 100% low tolerances were unsuccessful. Possibly, this failure was due in part to the high incidence of sterility in the animals exhibiting the poorest tolerance. Our Wistar (W) colony has essentially 100% normal tolerance curves. A comparison of the so-called normal curves from the Y strain with those from the W strain show that they have the same shape, but that the Y strain blood sugars are higher throughout the 5-hour observation (3).

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Matings. (*A*) Low tolerant ϒ males were crossed with: (*a*) low tolerant ϒ females, (*b*) normal ϒ females, (*c*) *W* females. (*B*) *W* males were crossed to low tolerant ϒ females. (C) Normal males and females from the F_1 generation of both types of ϒ and *W* crossings were mated.

RESULTS

All of the experimental animals of the ϒ stock used in this study were descendants of 46ϒ. In the families represented in this group there were 104 males and 57 females whose tolerances were known, and table 1 shows that in this group 71% of the males and 58% of the females gave low tolerances. From figures 1, 2 and 3, it may be seen that the incidence of diabetic-like curves in the ϒ strain was the same from the mating of low tolerant ϒ males with normal ϒ females as from the mating of 2 low tolerant animals, suggesting as do other data a dominant position for the male in the transmission of this diabetic trait.

TABLE 1. THE EFFECTS OF MATING STRAINS ϒ AND *W* ON THE GLUCOSE TOLERANCE OF GENERATION F_1

No. of animals	Sex and type of tolerance	Hours after glucose injection[1]	
		½	5
		"True" sugar in terms of glucose per 100 cc. of blood	
		mg.	mg.
	Stock from which ϒ rats were derived		
30	Males, normal	223±3.2	137±2.7
74	Males, diabetic	209	192
24	Females, normal	208±3.6	133±3.4
33	Females, diabetic	221	223
	Stock from which W rats were derived		
22	Males, normal	170±3.2	110±0.8
19	Females, normal	187±3.9	96±2.8
	Crosses from W mothers and ϒ fathers F_1		
36	Males, normal	198±4.8	112±1.9
8	Males, diabetic	158	133
47	Females, normal	219±3.0	118±1.7
6	Females, diabetic	227	168
	Crosses from ϒ mothers and W fathers F_1		
7	Males, normal	163	104
6	Males, diabetic	154	127
14	Females, normal	185	94
2	Females, diabetic	189	155

[1] 3.50 gm. of glucose per kg. in a 10% solution.

For the cross-breeding studies, ϒ rats were selected with typical or more marked than average decreased glucose tolerance and *W* rats were selected with a tolerance near the average for the group. Figures 3 and 4 show the results of the crossing of ϒ males 326 and 316 with ϒ and *W* females. With ϒ females, 32 males and 31 females were produced. 75% of these males and 58% of these females gave diabetic-like tolerances. The incidence of low glucose tolerances from ϒ male and *W* female crosses was 18% in the males and 11% in the females. In the reciprocal crosses shown in figure 5 the small number of animals probably invalidates the percentage of low tolerant animals which is higher for the males although the same for the females as in the crosses of the ϒ males and *W* females. Attention should be called to the fact that in both types of crosses the animals classed as low tolerant attain that classification by a narrow margin. Considering the actual blood sugar values, there is a highly signifi-

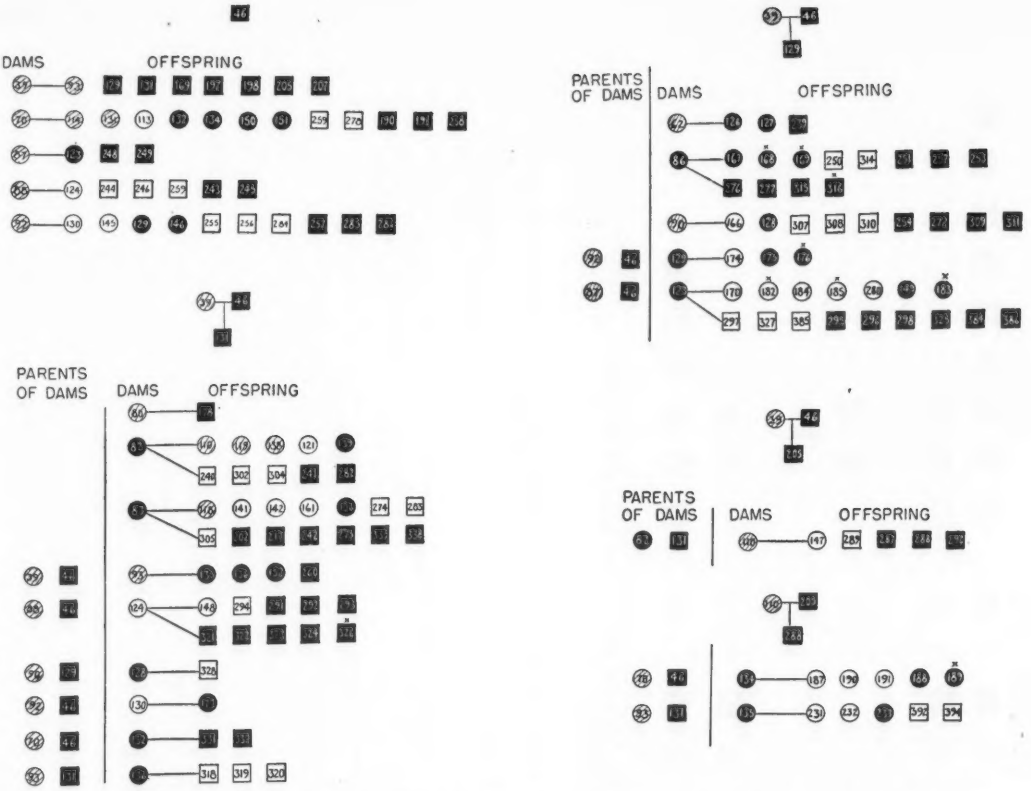

Fig. 1. Υ stock. Black squares and circles, diabetic type of glucose tolerance; white squares and circles, normal glucose tolerance; shaded squares and circles, tolerance not determined. ×—Animal used for cross breeding.

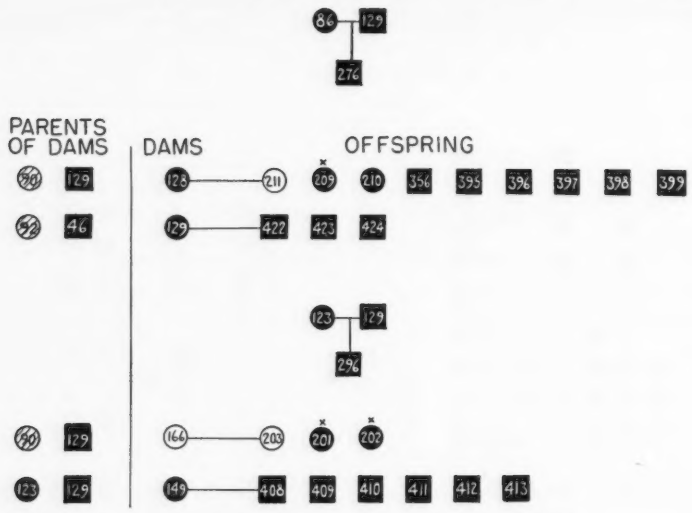

Fig. 2. ϒ stock. Black squares and circles, diabetic type of glucose tolerance; white squares and circles, normal glucose tolerance; shaded squares and circles, tolerance not determined. ×—Animals used for cross breeding.

TABLE 2. THE EFFECTS OF MATING NORMALS FROM GENERATION F_1 ON THE GLUCOSE TOLERANCE OF GENERATION F_2

No. of animals	Sex and type of tolerance	Hours after glucose injection[1]	
		½	5
		"True" sugar in terms of glucose per 100 cc. of blood	
	F_2 generation		
	Parents—♀ 20, ♂ 19 & ♂ 35		
12	Males, normal	163	92
7	Females, normal	216	111
	Parents—♀ 66, ♀ 67 & ♂ 53		
7	Males, normal	173	96
4	Females, normal	211	100
	Parents—♀ 70 & ♂ 55		
5	Males, normal	172	91
6	Females, normal	222	105
	Average F_2 generation		
24	Males, normal	167±3.2	92±2.0
17	Females, normal	217±3.5	106±4.3
	Results of crossing F_1 back to diabetic type ϒales		
	Parents—♀ 5x & ♂ 316ϒ		
2	Males, normal	197	126
5	Females, normal	265	121
	Parents—♀ 17x & ♂ 427ϒ		
1	Male, normal	199	115
4	Females, normal	180	96

[1] 3.50 gm. of glucose per kg. in a 10% solution.

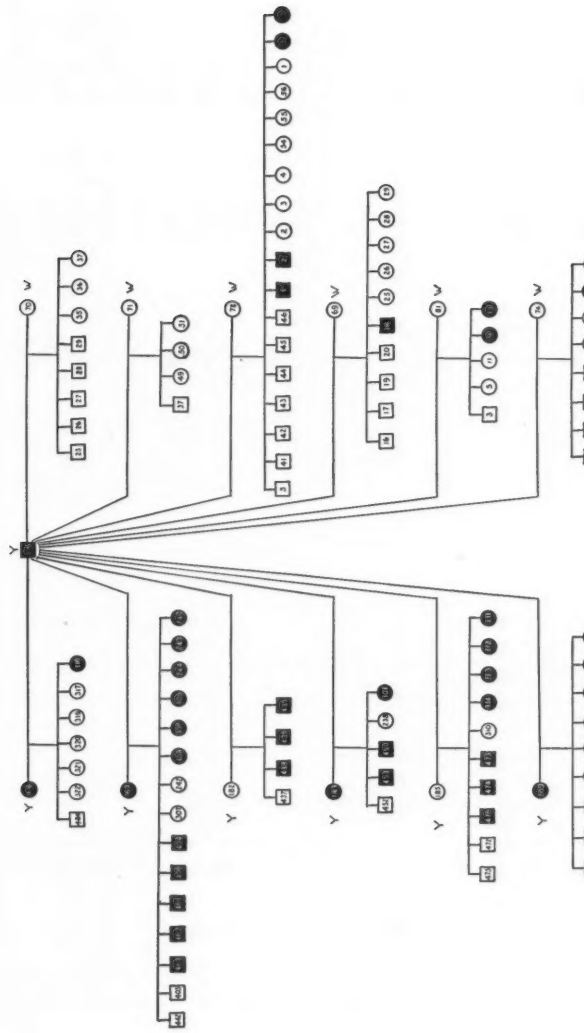

Fig. 3. 316Y crossed to Y and W females. Black squares and circles, diabetic type glucose tolerance; white squares and circles, normal glucose tolerance.

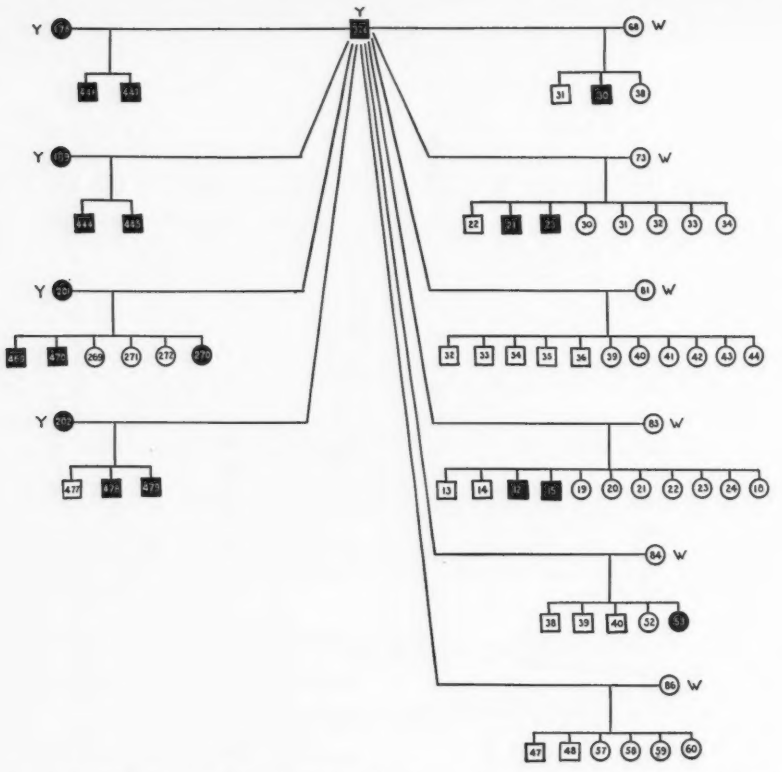

Fig. 4. 326Y crossed to Y and W females; Black squares and circles, diabetic type glucose tolerance; white square and circles, normal glucose tolerance.

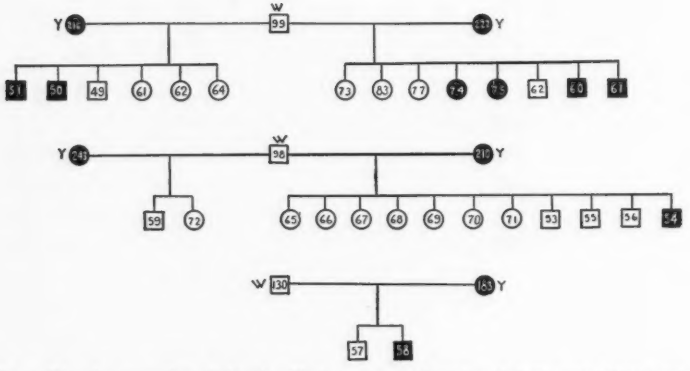

Fig. 5. W males crossed to Y females. Black squares and circles, diabetic type glucose tolerance; white squares and circles, normal glucose tolerance.

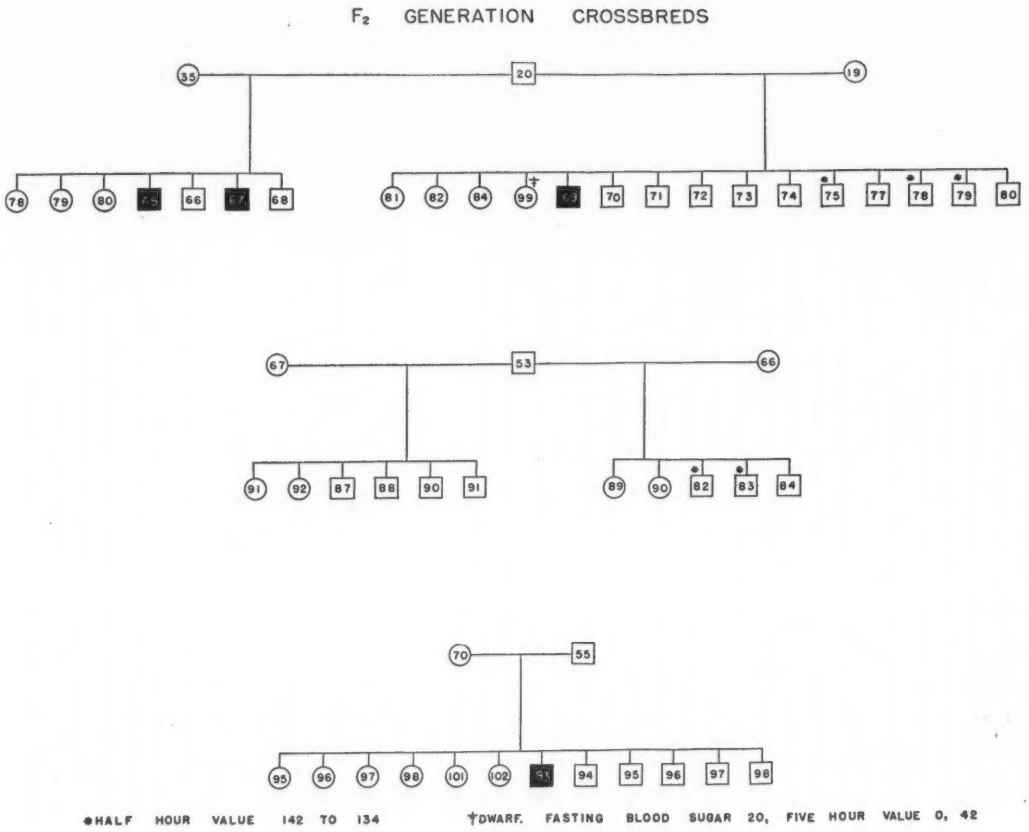

Fig. 6. F_2 generation. Black squares and circles, diabetic type glucose tolerance; white squares and circles, normal glucose tolerance.

cant difference between the crosses of Υ fathers to W mothers and W fathers to Υ mothers (table 2), again suggesting the greater importance of the male in the inheritance of glucose tolerance. It may be seen in table 1 that the normal females of the F_1 generation resemble the normals of the sires' stock. This is also true of the normal males from the cross of W sires to Υ dams. However, the normal males with Υ sires give a half-hour blood sugar midway between the Υ and W males, and a 5-hour value like that of the W males. It is obvious from table 1 that the severity of the decreased tolerance is markedly less in all of the crosses than in the Υ stock.

From the F_2 generation some further explanation of the mechanism of inheritance might be expected. Crossing only normal animals of the F_1 generation, we obtained 28 males and 17 females (fig. 6). Only 4 of these males showed a diabetic type of tolerance; 3 of these had F_1 parents from Υ male and W female crossing. Five males and one dwarf female gave unusually high tolerances. The dwarf also showed a low fasting blood sugar of 20 mg.% 15 hours after the withdrawal of food as compared with the average of 70 for the W strain after a similar fast. F_1 crossbred female 5 bred to her Υ father produced 7 offspring none of which gave a diabetic-like tolerance, but the half-hour value of the 5 females was very high (table 2). A cross between F_1 crossbred female 17 and a low tolerant Υ relative with low tolerant parents gave 5 offspring with normal curves very much like those obtained from the W strain (table 2). From tables 1 and 2 it may be seen that there is no significant difference in the half-hour values of the F_2 males and the W males, but the 5-hour value of the F_2 males is 18 mg.% lower than that of the W males. The chance that this difference is not significant is less than one in a million. In the females of the F_2 generation there is no significant deviation from the Υ stock on the half-hour, but on the fifth hour the F_2 value is much lower, approaching closely that of the W stock. The chances that the fifth hour values for the F_2 and Υ stock are the same are less than one in a thousand, but for the F_2 and W stock, nineteen in a hundred.

SUMMARY

The inheritance of glucose tolerance has been studied by crossing a Υ strain family in which 71% of the males and 58% of the females gave diabetic-like glucose tolerances with a W strain family showing no evidence of low tolerance. The families employed were typical for the respective strains. Crossing the Υ and W strains gave an F_1 generation whose diabetic-like glucose tolerance was less than that of the Υ stock in both incidence and severity.

The sire appears to exert a greater influence than the dam on the type of glucose tolerance of the offspring.

In the F_2 generation from normal F_1 animals the incidence of decreased tolerance in the males was only 14% and 0 in the females. Four individuals showed a glucose tolerance greater than that of the W strain. The appearance of these hypertolerant F_2 animals suggests new combinations of hypoglycemic genetic factors.

The decreased glucose tolerance is not a simple recessive but is probably the result of one principal gene plus modifiers, the principal gene tending to be incompletely recessive. The penetrance of this gene is less than 100% in homozygous combinations.

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SYNERGISM OF THE FOLLICLE STIMULATING AND LUTEINIZING HORMONES IN PRODUCING ESTROGEN SECRETION[1]

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THE PITUITARY gonadotropic hormones (FSH and LH) have been shown to be complementary in ovarian development and in the production of secretion of male hormone from the testes. This paper extends the observations previously reported on the synergism of the two factors in the initiation of secretion of estrogen from the ovaries of rats (1). It has been generally believed that FSH is the factor which initiates estrogen secretion from the follicles whose growth it stimulates. While this may be essentially true it has been found that LH in conjunction with FSH increases the secretion of estrogen over that produced by FSH alone. LH alone, on the other hand, is inactive for this reaction in the immature ovary.

MATERIAL AND METHODS

The FSH and LH used in these experiments were prepared from sheep pituitaries by methods previously reported (2). The purified and concentrated extracts were dried over phosphorous pentoxide in vacuo at 0°C. and the powders standardized and stored. Such powders have been found to retain their activity for at least 2 years.

The preparations were injected as fine emulsions of the tannic acid precipitates and unless otherwise stated the injections were made subcutaneously. The duration of injections varied from 2 to 4 days as indicated, injecting twice daily 0.25 cc. per injection. When both FSH and LH were given simultaneously they were injected separately. At the end of the experiment the ovaries and uteri were removed and weighed. The uteri were opened longitudinally and all excess moisture removed by spreading on absorbent paper.

RESULTS

The results are presented in tables 1 to 6. Each figure in the tables represents the average of data obtained from at least 6 animals.

FSH alone may increase the ovarian weight 200%, due to follicular development with very little estrogen secretion taking place as shown by the uterine weights when the injection period is 48 hours. With increasing dosage, however, uterine growth takes place indicating increased secretion of estrogen (table 1).

The injection of LH in addition to the FSH results in pronounced uterine hypertrophy even when an amount of FSH is given which alone produces neither ovarian nor uterine growth (table 2). The amount of LH necessary to elicit the increase in estrogen output is very small. The LH preparation used in these experiments contained 40 male R.U. per mg. but 0.0008 mg. was sufficient to produce a 90% increase in uterine weight when combined with an amount of FSH which caused no uterine enlargement.

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TABLE 1. ESTROGEN SECRETION INDUCED IN NORMAL IMMATURE RATS, 21 DAYS OLD, WITH FSH INJECTIONS FOR 2 DAYS

Amount injected	Ovarian weight	Ovarian increase	Uterine weights	Uterine increase
mg.	mg.	%	mg.	%
0.00	9	—	21	—
0.01	16	60	24	—
0.02	20	100	22	—
0.04	26	160	20	—
0.10	32	220	24	—
0.20	30	200	28	40

TABLE 2. ESTROGEN SECRETION INDUCED IN NORMAL IMMATURE RATS, 21 DAYS OLD, WITH FSH AND LH INJECTIONS FOR 2 DAYS

Amount injected FSH	Amount injected LH	Ovarian weights	Ovarian increase	Uterine weights	Uterine increase
mg.	mg.	mg.	%	mg	%
none	2	11	none	18	none
0.025	none	18	80	20	none
0.025	0.025	24	140	49	195
0.025	0.012	19	90	45	125
0.025	0.006	20	100	48	140
0.012	none	13	30	18	none
0.012	0.025	21	110	56	180
0.012	0.012	15	50	46	130
0.012	0.003	16	60	54	170
0.012	0.0008	13	30	38	90
0.006	none	11	10	39	none
0.006	0.025	15	50	48	140
0.006	0.012	12	20	30	50
0.006	0.006	12	20	23	none

TABLE 3. ESTROGEN SECRETION IN NORMAL IMMATURE RATS RESULTING FROM 4 DAYS INJECTIONS OF FSH

Amount injected	Ovarian weight	Ovarian increase	Uterine weights	Uterine increase
mg.	mg.	%	mg.	%
0.02	18	80	26	30
0.04	24	140	32	60
0.08	32	220	47	135
0.16	35	250	44	120
0.50	44	340	63	215

TABLE 4. ESTROGEN SECRETION IN NORMAL IMMATURE RATS RESULTING FROM 4 INJECTIONS OF FSH AND LH

Amount injected FSH	Amount injected LH	Ovarian weights	Ovarian increase	Uterine weights	Uterine increase
mg.	mg.	mg.	%	mg.	%
	1.0000	11	0	18	0
0.02		17	70	24	20
0.02	0.0250	28	180	72	260
0.02	0.0100	24	140	82	310
0.02	0.0050	20	100	66	230
0.02	0.0025	18	80	60	200
0.02	0.0010	16	60	49	145

Gross histological examination of the ovaries from this series of experiments revealed no differences in those produced with FSH and those resulting from FSH and LH. The uteri when enlarged were typically estrous, the larger ones being characteristically 'balloon-like' in appearance.

FSH alone produced greater uterine hypertrophy in proportion to ovarian growth when the injections were continued over a period of 4 days then was true in the 2-day series. However, the uterine enlargement was not great except with the largest dose of FSH given, being in all cases less than the ovarian increase when calculated on a percentage basis (table 3).

TABLE 5. ESTROGEN SECRETION IN HYPOPHYSECTOMIZED IMMATURE RATS INDUCED WITH FSH INJECTIONS FOR 4 DAYS

Amount injected	Ovarian weights	Ovarian increase	Uterine weights	Uterine increase
mg.	*mg.*	*%*	*mg.*	*%*
0	6	—	11	—
0.02	10	66	12	0
0.04	16	166	8	0
0.08	24	300	15	50
0.20	31	400	17	70
0.50	35	480	27	170

TABLE 6. ESTROGEN SECRETION IN HYPOPHYSECTOMIZED IMMATURE RATS INDUCED WITH FSH AND LH INJECTIONS FOR 4 DAYS

Amount injected		Ovarian weights	Ovarian increase	Uterine weights	Uterine increase
FSH	LH				
mg.	*mg.*	*mg.*	*%*	*mg.*	*%*
0.02	0	12	100	11	none
	1.000	8	none	9	none
0.02	0.100	24	300	42	320
0.02	0.050	20	230	44	340
0.02	0.025	17	180	33	230
0.02	0.010	13	100	20	100

A marked increase in estrogen secretion over that produced by FSH alone resulted when both FSH and LH were given. The results of the 4-day injections were therefore similar in all respects to those of the 2-day experiments (table 4).

Rats which were hypophysectomized on the 25th day of life were injected with FSH and with FSH and LH for 4 days beginning 3 days after the operation (table 5, 6). The results were similar to those obtained in normal immature rats. FSH produced marked ovarian enlargement with relatively small uterine increase. LH when combined with FSH had the same augmenting effect on estrogen secretion as shown in normal animals.

Experiments in which FSH and LH were injected in sequence instead of simultaneously were carried out but in none of these experiments was estrogen secretion increased significantly.

DISCUSSION

It appears that both FSH and LH are concerned in the secretion of estrogen from the ovaries of the rat. FSH however plays the primary role in that it is capable of initiating the secretory activity. A secondary role is played by LH since it alone cannot stimulate the secretory mechanism to activity but merely increases the effectiveness of

the FSH. The situation is therefore the reverse of that which obtains for the stimulation of the secretory activity of the male gonad where the LH is the primary factor and FSH is the augmenting agent (3).

The possibility presents itself that FSH, if completely free of LH, may stimulate the development of the follicles without initiating secretion of estrogen. As shown by the results, very small amounts of LH are necessary to produce a marked increase in estrogen secretion. The slight secretion of estrogen induced by the FSH preparations may therefore be due to the presence of traces of LH which cannot be detected by other methods. While the FSH preparations do not produce luteinization or induce secretion of male hormone from the testes (physiological reactions due to LH) they may still contain amounts of LH subminimal for these reactions. From the results presented in this paper, however, we can conclude only that FSH is relatively inefficient in the induction of estrogen secretion as compared to FSH plus LH.

The use of uterine weights as a method of standardizing pituitary extracts (4) is open to the same objections that have been raised with regard to ovarian weights as an endpoint. With unfractionated extracts the weights obtained do not reflect the amount of either one of the hormones present in the extract but depend on the relative proportions of the 2. The results from one extract to the next cannot therefore be compared. With 'pure' FSH on the other hand the ovaries are more sensitive and respond more quickly than do the uteri which are comparatively unresponsive. On the other hand the marked increase in estrogen secretion with resulting increase in uterine weight when LH is injected with FSH, may serve as a quantitative measure of the luteinizing hormone. This possibility is being investigated at the present time.

SUMMARY

The follicle stimulating hormone was relatively inefficient in inducing the ovaries of immature or hypophysectomized rats to secrete estrogen.

The luteinizing hormone, while unable to stimulate the ovaries to secrete estrogen, markedly increased secretory activity when injected with FSH.

Increase in uterine weight is not a reliable endpoint for FSH assay unless the FSH is free of LH. When this is true the ovaries are more sensitive than are the uteri.

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SYNERGISM OF ESTROGENS WITH PITUITARY GONADOTROPINS IN HYPOPHYSECTOMIZED RATS[1]

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IT HAS BEEN KNOWN ever since the pioneer work of Walsh, Cuyler and McCullagh in 1934 (1) that spermatogenesis in the rat could be maintained after hypophysectomy by the prompt and continued injection of male sex hormone. No corresponding maintenance of ovarian function by estrogenic substances had been observed until recently when Williams (2) reported an increase in the weight of ovaries of hypophysectomized rats implanted with diethylstilbestrol, and an augmentation of the gonadotropic effect of pregnant mare serum in animals simultaneously receiving stilbestrol. Pencharz (3) has also noted an increased ovarian size due to enlargement of follicles following the treatment of hypophysectomized rats with stilbestrol and other estrogens. Even more interesting is his observation of great augmentation of ovarian weights and the formation of large follicles and corpora lutea in animals given certain estrogens together with chorionic gonadotropin. The augmentation of this gonadotropin is even more significant of the effect of stilbestrol on the ovary than the augmentation of pregnant mare serum hormone by stilbestrol reported by Williams because of the very limited nature of the stimulation given the hypophysectomized rat's ovary by chorionic gonadotropin.

No difficulty was experienced in confirming the striking discovery of Pencharz that certain estrogens cooperate with chorionic gonadotropin in producing changes in the ovaries of hypophysectomized animals which either material alone is unable to effect (table 1). It will be noted that the ovaries of hypophysectomized animals treated by the stilbestrol from time of operation were definitely larger than those of hypophysectomized controls 9 days after operation (23 to 38 mg. vs. 10 mg. in controls). This enlargement was due to slight but definite growth of many follicles (fig. 4). The follicles in spite of somewhat increased size did not develop antra. Another peculiarity of the follicles was a peripheral zone of degeneration. The interstitial tissue of the ovary was not repaired in the stilbestrol treated animals. The amount of interstitial tissue was small, probably due to the checking of follicular atresia. The ovaries of hypophysectomized animals injected with chorionic gonadotropin (8 R.U.) were also slightly increased in weight (16 to 26 mg.), the increase being due to interstitial tissue hypertrophy, no follicular growth being present (fig. 2). The ovaries of the animals receiving both stilbestrol and chorionic gonadotropin were much larger than could be expected on the basis of additive effects, the weights ranging between 75 and 111 mg. The great increase in size of the ovaries in this case

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TABLE 1. EFFECT OF CHORIONIC GONADOTROPIN[1] IN HYPOPHYSECTOMIZED FEMALE RATS[2] TREATED WITH DIETHYLSTILBESTROL[3]

Material injected	Total dose (subc.) mg./rat	Ovaries		Uterus+Oviducts	
		Weight	Histology	Weight	Description
Stilbestrol	8	mg. 27 38 23 28 Ave. 29	Many sF (without antra, zone of degeneration of granulosa near periphery); IT deficient	mg. 215 252 182 211 Ave. 215	est
Antuitrin-S	0.5 (10 R.U.)	26 22 21 16 21	No F development; IT repaired	38 27 32 31 32	s
Stilbestrol + antuitrin-S	8 + 0.5	75 105 111 73 91	lF, CL, cysts, with thecal luteinization of cysts; IT repaired	142 146 152 175 154	l
Controls	—	10 (Ave. 17 rats)	No F development; IT deficient	26	s

[1] Antuitrin-S kindly supplied by Parke, Davis & Company.

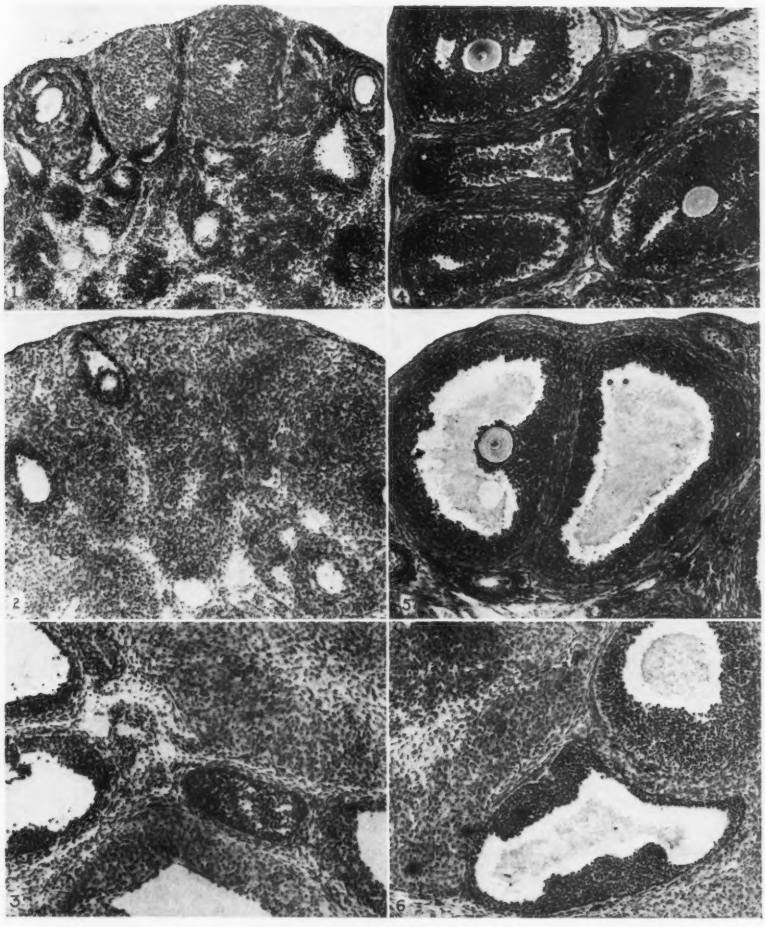
[2] Rats 28 days of age at operation, stilbestrol injections begun 1 day after operation and continued for 8 days. Antuitrin injected from the 6th to the 8th day postoperative. Autopsy 72 hours after onset of antuitrin injection or on 9th day postoperative. Only completely hypophysectomized rats included in this and other tables.

[3] Diethylstilbestrol kindly supplied by Squibb & Sons; injected dissolved in sesame oil.

TABLE 2. EFFECTS OF HYPOPHYSEAL ICSH AND FSH IN HYPOPHYSECTOMIZED FEMALE RATS[1] TREATED WITH DIETHYLSTILBESTROL

Material injected	Total dose mg./rat	Ovaries		Uterus+Oviducts	
		Weight	Histology	Weight	Description
ICSH	0.080 (8 R.U.) IP	mg. 16 15 8 10 10 10 Ave. 12	No F dev; IT repair	mg. 35 30 33 25 24 23 Ave. 28	s
FSH	0.024 (8 R.U.) subc.	29 32 41 39 50 38	m, lF; IT deficient	145 91 115 95 74 104	est
Stilbestrol	10 mg. pellet subc.	39 28 40 14 21 28	s to smF, no antrum, zone of degeneration of granulosa near periphery; IT deficient	199 183 240 115 194 186	est
Stilbestrol + ICSH	as above	32 30 41 30 33	s, smF, more irregular in size, perhaps fewer, zone of degeneration less evident; IT repair	197 259 214 202 218	est
Stilbestrol + FSH	as above	144 109 157 87 113 120 122	lF, CL; thecal luteinization of lF; IT deficient	186 182 190 217 178 248 200	l or est
Controls	—	10 (Ave. 17 rats)	No F dev; IT deficient	27	s

[1] Rats 27 days of age at operation; diethylstilbestrol pellet implanted 1 day postoperative, removed at autopsy (day 9); ICSH and FSH injected day 5–7, animals autopsied 96 hours after first injection or on 9th day.



OVARIES OF HYPOPHYSECTOMIZED RATS (×70) Fig. 1. Control (27 days of age at operation, 9 days post-operative) 2 ovaries = 12 mg. Fig. 2. Treatment 10 R.U. antuitrin-S in 3 daily subcutaneous injections. Autopsy 72 hr. (9 days postoperative). 2 ovaries = 16 mg. Fig. 3. Treatment 8 mg. diethylstilbestrol in sesame oil (injected subcutaneously, ⅓ cc. 3 times daily for 8 days) and 10 R.U. antuitrin-S injected subcutaneously days 6 to 8 inclusive. Autopsy day 9. 2 ovaries = 73 mg. Fig. 4. Treatment 10 mg. pellet diethylstilbestrol implanted subcutaneously and allowed to remain until autopsy on the 9th day. 2 ovaries = 50 mg. Fig. 5. Treatment 8 R.U. FSH in 3 daily subcutaneous injections days 5–7 of experiment. Autopsy 96 hours after onset of injection, or day 9. 2 ovaries = 50 mg. Fig. 6. Treatment 10 mg. pellet diethylstilbestrol and 8 R.U. FSH administered as above. 2 ovaries = 157 mg.

was due to the development of large follicles, thecal luteinized follicles and corpora lutea as well as interstitial tissue response (fig. 3).

Alpha-estradiol dipropionate (Ciba) was not effective in synergizing similar doses of chorionic gonadotropin when implanted as 10 mg. tablets and allowed to remain over the same period.

We were naturally greatly interested in knowing whether either of the two

gonadotropic fractions from the pituitary would similarly be synergized by large doses of stilbestrol. For this reason hypophysectomized rats were given the estrogen simultaneously with purified ICSH or purified FSH.[2] As will be seen in table 2, 10 mg. tablets of stilbestrol were implanted subcutaneously the day after hypophysectomy. When the animals were killed 9 days postoperatively, the ovaries were found to weigh 28 mg.; those of controls at this postoperative period weighed 10 mg. The ICSH, injected alone from day 5 to 7 postoperative, produced 12 mg. ovaries, the ovaries showing interstitial repair but no follicular development. Combinations of stilbestrol and ICSH produced ovaries of a size anticipated on the basis of the additive effects of the two substances. Histologically, the ovaries showed the separate effects of the two substances. There were, therefore, no synergic effects between the two substances at the particular doses administered.

TABLE 3. EFFECTS OF HYPOPHYSEAL ICSH AND FSH IN HYPOPHYSECTOMIZED MALE RATS[1] TREATED WITH DIETHYLSTILBESTROL

Material injected	Total dose mg./rat	Testes			Seminal Vesicles			Prostate		
		mg.		Ave.	mg.		Ave.	mg.		Ave.
ICSH	0.100 (10 R.U.) IP	206			7			41		
		347			8			71		
		271			10			56		
		399		306	12		9	15		46
FSH	0.024 (8 R.U.) subc.	343	310		10	7		36	34	
		380	335		8	8		42	35	
		418	442	371	10	11	9	47	40	39
Stilbestrol	10 mg. pellet subc.	310	139		14	17		51	35	
		225	189		11	12		45	25	
		130	192	198	17	14	14	35	40	39
ICSH+Stilbestrol	as above	172	363		14	14		36	53	
		308	309		16	13		59	48	
		158		262	10		13	36		46
FSH+Stilbestrol	as above	338	349		15	17		39	35	
		438	252		16	26		34	55	
		442		364	13		16	41		40
Controls	—	147	138		8	7		32	32	
		212	206		8	8		40	35	
		220	98	170	9	11	9	35	34	35

[1] Rats 27–31 days of age at operation; diethylstilbestrol pellet implanted one day postoperative removed at autopsy (day 11); ICSH, FSH injected days 6–10.

The dose of FSH used caused the development of 29 to 50 mg. ovaries, the enlargement being due entirely to the development of medium and large follicles; the interstitial tissue remained deficient (fig. 5). When this dose of FSH was given with stilbestrol, the ovaries weighed 87 to 157 mg. and contained large follicles, corpora lutea, and thecal luteinized follicles (fig. 6). The interstitial tissue was not repaired (or only partially). The synergic effect of stilbestrol was therefore with the hypophyseal follicle stimulating fraction rather than with the interstitial cell stimu-

[2] The preparation of the two gonadotropic fractions has already been reported ("Purification of the follicle-stimulating hormone of the anterior pituitary." H. L. Fraenkel-Conrat, M. E. Simpson and H. M. Evans. Apartado de los "Anales de la Facultad de Medicina" Montevideo, XXV, Nos. 5, 6, 7 y 8, 1940. "Interstitial cell stimulating hormone. II. Method of preparation and some physico-chemical studies." Choh Hao Li, Miriam E. Simpson and Herbert M. Evans. *Endocrinology*, in press).

lating fraction as anticipated because of the similarity of the last mentioned substance with chorionic gonadotropin.

Doses of FSH and ICSH similar to those reported here in females were also given to hypophysectomized male rats, both alone and in combination with stilbestrol but no synergic effects were observed (table 3).

SUMMARY

The synergic action of stilbestrol with chorionic gonadotropin in bringing about development of the ovaries of hypophysectomized rats has been confirmed.

Two hypophyseal gonadotropic fractions (FSH and ICSH) have been tested for this synergic action with stilbestrol and the synergism has been shown to exist in the case of stilbestrol and FSH.

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RETARDATION OF MAMMARY INVOLUTION IN MICE BY INJECTION OF LACTOGENIC HORMONE[1]

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EXPERIMENTS utilizing suckling young (1–4) and the substitution of a chemical irritant for the suckling young (5) have shown that stimulation of the nipple is a major factor in maintaining lactation and in preventing mammary involution in rats and mice. The chain of events initiated by stimulation of the nipple has not been elucidated, however. It appears most likely that the central effect of such stimuli is upon one or more of the endocrine glands which, in turn, acts upon the mammary gland. The prompt cessation of lactation following hypophysectomy in several species (6) suggests that the hypophysis is the gland affected. And, indeed, it has been reported (7) that suckling alters the lactogenic hormone content of the hypophysis in rats.

If the effective consequence of stimulation of the nipples in the lactating animal is release of pituitary lactogen, it follows that administration of this substance should retard mammary involution in females isolated from their young. The experiments reported here were conducted to determine whether such is the case.

MATERIALS AND METHODS

Young adult mice of the Strong NH strain were used. In order that lactation might become established before experimental intervention, all the animals were allowed to remain undisturbed for 4 days after parturition with litters regulated at 6 members. In most instances the animals were primipara, although a few were in the second period of lactation.

At the end of the fourth day post-partum, 12 lactating females were isolated from their young and started immediately on daily subcutaneous injections of a lactogenic extract of anterior hypophysis.[2] Seven of these animals were given daily injections of 2 mg. in a single dose, and one animal received 2 injections daily of 1 mg. each. Two animals received 3 mg. daily, and 2 were given 1 mg. daily. Two of the animals receiving 2 mg. daily were ovariectomized immediately upon isolation from their young. All of the animals were autopsied after 7 days of treatment. For comparison 9 untreated females of otherwise identical history were killed for study 7 days after segregation from their young.

At autopsy the mammary glands were removed with the skin and fixed in Bouin's fluid. After the glands were freed from the skin they were stained in Mayer's haemalum. The stained glands were dissected from adventitial connective tissue and muscle, cleared and mounted on slides in damar. The inguinal glands were used for

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[1] This study was aided by a grant from the Fluid Research Fund of Yale University School of Medicine.

[2] Generously supplied by Difco Laboratories through the courtesy of Dr. J. P. Schooley. This material was received as a powder containing 20 I.U. of prolactin per mg. For injection the powder was dissolved in a concentration of 10 mg. per cc. in water at PH 8.

histologic sections. Sections were also made of the ovaries, uteri and adrenals. These organs were fixed in Bouin's fluid and stained with Ehrlich's hematoxylin and triosin.

RESULTS

The principle features of mammary involution after a lactation period of 4 days have already been described (5), but for purposes of orientation they may be reviewed briefly. As a result of the interruption of suckling there is first an engorgement of the glands and a subsequent rapid diminution of milk content. On the third day after removal of the young the glands are morphologically intact despite a remarkable loss of retained secretion. From this point onward there is a rapid regression of the parenchyma. The alveoli collapse forming frayed solid masses of cells. As a result of disintegration of the smaller ducts these masses often become isolated from the duct system to the extent that the mammary glands come to be represented by widely separated islands of small cells. The terminal stage appears to be a fatty change, with the former site of alveoli and terminal ducts being occupied by small fat corpuscles. The main duct system exhibits a slower degeneration, beginning on the fifth day. The earliest change is diminution in the height of the lining epithelium and a concomitant decrease in vacuolation of these cells. After 7 days these cells exhibit considerable pyknosis and much sloughing into the lumen of the duct.

The mammary glands of the 9 untreated animals 7 days after removal of the young presented a remarkable uniformity in the extent of involution. The alveoli were represented merely by widely scattered, solid islands of small, rounded cells in no respect like gland cells (fig. 1). In these masses cell boundaries were poorly defined and the nuclei were predominantly pyknotic. The lobular arrangement of the glands was almost entirely lost (fig. 2), although the main ducts were preserved except for the epithelial degeneration mentioned above.

In the animals receiving the lactogenic hormone, on the other hand, mammary engorgement persisted for at least 3 days after removal of the young, although it was impossible to determine precisely when the engorgement subsided. At autopsy the mammary glands of all of the treated animals were more hyperemic than in the untreated animals, although the degree of hyperemia was not the same in all the members of the former group. There was considerable variation in the extent of involution after 7 days of treatment, but the general average stage of involution when all the cases are considered was less than the degree of regression occurring in 4 days in the absence of treatment.

Ten of the treated animals exhibited quite evident retardation in mammary involution. In 4 instances the extent of regression was significantly less than that expected after only 3 days, in 4 cases the regression was the equivalent of 4 days, and in 2 the mammary glands were like those of an untreated animal 5 days after isolation from her young. The 2 remaining animals presented less retardation; in whole mounts the glands exhibited approximately 6-day involution, although the sections of the inguinal glands revealed no appreciable retardation.

In the 8 animals with mammary glands showing the most marked retardation of regression (the equivalent of 3 and 4 days) the whole mounts revealed an essentially undisturbed and intact duct system (fig. 3). The lobular structure was preserved, with the alveoli intact and showing no fraying which typically sets in on the fourth day. In every particular these glands were very similar to the mammary glands of the mouse at parturition.

The presence of many well preserved alveoli (fig. 4, 5) in the sections from these 8 animals was in itself evidence of significant retardation of involution. Although the number of such alveoli was relatively high in comparison with the condition

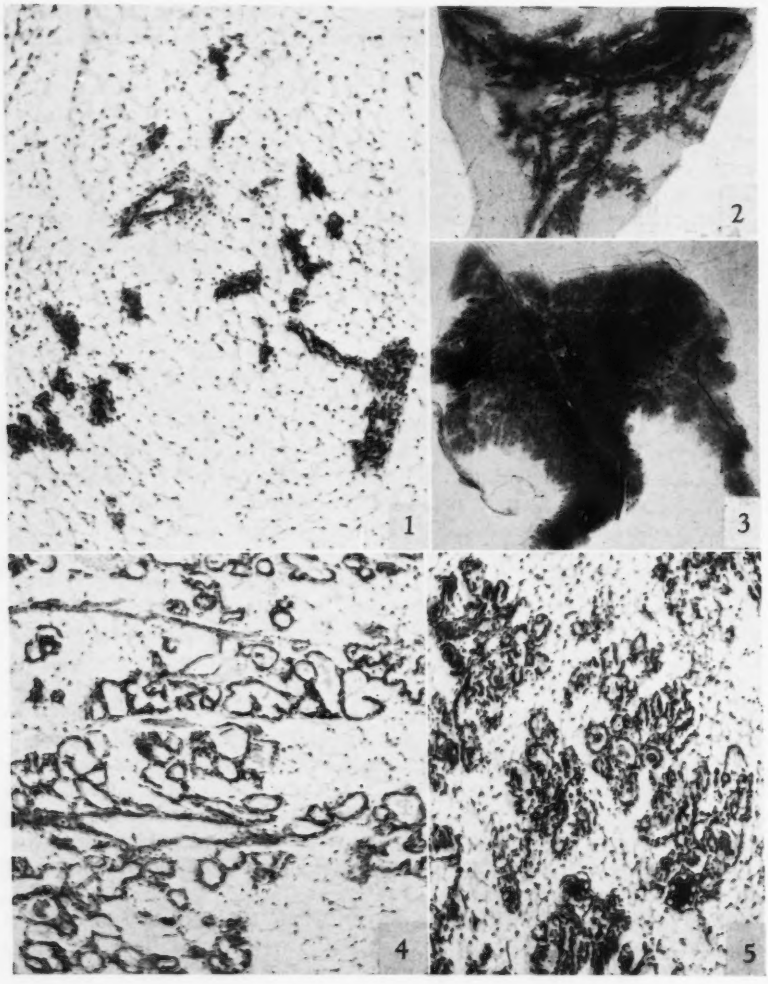

Fig. 1. SECTION OF A MAMMARY GLAND OF A MOUSE 7 DAYS AFTER REMOVAL OF THE YOUNG. The islands of cells which are remnants of alveoli and ducts persist in an abundant fatty stroma. ×100. Fig. 2. A MAMMARY GLAND 7 DAYS AFTER REMOVAL OF THE YOUNG. The ducts and small parenchymal islands as seen in figure 1 are shown. Whole mount. ×3.5. Fig. 3. A MAMMARY GLAND FROM A MOUSE UNSUCKLED FOR 7 DAYS. During this period the animal received 40 I.U. of prolactin daily. This gland is essentially like a gland at parturition. Compare with figure 2. Whole mount. ×3.5. Fig. 4, 5. SECTIONS OF MAMMARY GLANDS FROM 2 MICE WHICH RECEIVED DAILY INJECTIONS OF PROLACTIN FOR THE 7 DAYS FOLLOWING ISOLATION FROM THEIR YOUNG. Many alveoli are well preserved and several contain secretion. The usual signs of active involution apparent in untreated mice are minimal, however, the relatively small amount of parenchymal tissue indicates that some of the alveoli have completely regressed and have been replaced by fatty stroma. ×100.

characteristic of the control glands, the large amount of fatty stroma would indicate the complete regression of some of the parenchymal elements. These residual alveoli were smaller than the typical alveolus during full lactation and appeared to be in a

somewhat contracted state. Their epithelial lining was composed of tall cells with vesicular nuclei and a very abundant, granular cytoplasm, particularly in the apical portion as is characteristic of actively secreting mammary cells. Such non-dilated alveoli lined by tall epithelial cells of this type are of frequent occurrence during full lactation, but they by no means predominate in the latter condition. Many of the alveoli contained secretion products, but no appreciable congestion was apparent. The ducts were well maintained and exhibited no evidence of degeneration or marked congestion.

Several other features characteristic of regressing mammary glands were minimal in these glands. The so-called colostrum corpuscles were rare, as were the eosinophilic concretion-like masses which are of frequent occurrence in the ducts during involution. In addition, the number of round cell phagocytes, probably histiocytic in character, was fairly small.

In both whole mounts and sections the glands of the other 4 animals graded between those just described and mammary glands after a full 7 days' uninterfered involution.

Within the range of doses employed, the extent of retardation appears to be independent of the amount of lactogen administered. The mammary glands of an animal receiving 1 mg. daily were in all respects like glands after 2 or 3 mg. daily, either in single or divided doses. Moreover, ovariectomy seemed to have no influence upon the degree of retardation effected by the lactogen.

The side-effects of the injected material also appeared to be independent of the dose. In all cases the injected animals underwent a drastic change in temperament, becoming hyper-irritable and nervous. At autopsy considerable inflammation was present at the site of injection—most of the injections were of necessity made at the same site because the 'jumpiness' of the animal required that it be held for injection.

The ovaries of the treated animals were large and contained many corpora lutea and small follicles. The uteri of the intact, treated animals were very hyperemic and fairly large. Histologically these uteri revealed stromal evidence of progesterone stimulation (8) while the estrogen effects were minimal in character. The uteri of the ovariectomized, treated animals were in every respect castrate in type except for a moderate hyperemia.

The adrenals of the injected animals showed neither atrophy nor morphologic evidence of stimulation. They were identical with adrenals of normal, young adult females after breeding; i.e., the x-zone had disappeared.

DISCUSSION

In most instances the injected prolactin was effective in significantly retarding mammary involution. The principle effect of the hormone appeared to be maintenance of the integrity of lobule-alveolar and duct systems. The amount of secretion in the glands was not great, although sufficient to be significant. The alveoli and their cellular components, especially the tall lining epithelial cells whose apical portions were distended with granular material, were strikingly similar to many of the alveoli found during full lactation. The absence of cellular necrosis, sloughing of the epithelial lining of alveoli and ducts, as well as minimal colostrum cell formation and phagocytosis constitute additional evidence of retardation of involution. The failure of the prolactin in all of the cases to maintain full lactation may be due, as has been suggested (9, 10) to the non-removal of the products of secretion, especially during the early part of the injection period.

The questionable retardation of involution in 2 of the animals was perhaps re-

lated to poor development of the mammary glands at the start of treatment. It is interesting, at any rate, that at the end of the 4 days of suckling preceding treatment the young in these 2 instances were thin and little milk was in their stomachs.

The observations that both stimulation of the nipples with a chemical irritant (5) and injections of lactogenic hormone retard mammary involution in mice isolated from their young indicate that the effect of suckling is mediated by stimulation of the nipple which in turn evokes the production and release of lactogen, which in its turn maintains the mammary gland. It is possible, however, that nipple stimulation provokes activity in other endocrine glands which influence the mammary apparatus. The persistence of full lactation in ovariectomized animals is well known and serves to exclude the ovary as a significant link in the chain. The adrenal cortex has been reported to be involved in the initiation and maintenance of lactation (11, 12) but this involvement has not been shown to be direct (13). It is likely that the adrenal cortex, the pancreas, the thyroid and the parathyroids are all concerned in maintaining a bodily economy which will, among other things, support lactation; but their influence does not appear to be upon mammary activity specifically. On the other hand, the lactogenic hormone preparation used in these experiments had a decided effect in retarding involution. The adrenals of the treated animals did not show any morphologic evidence of stimulation, and any relevant action of the extract upon the ovaries is excluded by the similarity of the mammary glands of the intact and ovariectomized mice. Thus the effect of the extract must apparently be attributed solely to its lactogenic hormone.

There are few observations regarding the relation of lactogenic hormone to the mechanism under consideration recorded in the literature. Grant (14) has reported a meager and transient restitution of lactation in partially regressed mammary glands of guinea pigs receiving prolactin. The regression itself appears to have been somewhat retarded, however. The observations (7) that suckling modifies the lactogenic hormone content of the rat's hypophysis rather definitely implicates this hormone in the suckling 'reflex'. The involvement of the hypophysis in lactation in the mouse has not been satisfactorily analyzed. Aside from the termination of lactation by hypophysectomy (15) there seem to be no appreciable effects of lactogenic hormone reported for this species (6).

The present observations may be interpreted to mean that stimulation of the nipples results in release of lactogenic hormone which retards mammary involution. The mediation of the stimulation upon the hypophysis, however, is an open question. That it is by way of the nervous system is indicated by the experiments of Inglebrecht (16) who found in rats that spinal transection between the last thoracic and first lumbar segments led to involution of all the mammary glands if only the posterior group of glands was suckled, while suckling of the pectoral glands resulted in maintenance of all the glands. The spinal transection, according to Inglebrecht, produced loss of sensation in the posterior group of glands but did not interfere with the anterior glands. The entrance of the effector nerve impulse or its effects into the anterior lobe of the hypophysis offers many problems in view of the difficulty in understanding hypophyseal innervation which persists despite the not inconsiderable study it has received.

SUMMARY

Of 21 lactating mice isolated from their young on the fourth day postpartum, 12 were given 7 daily injections of 20 to 60 I.U. of prolactin and 9 were untreated. Compared with the untreated controls, 10 of the animals receiving prolactin exhibited definite retardation in mammary involution. The average involution in the treated animals was that of 4 days in contrast to the 7 days of segregation from their young.

The inhibition of involution manifested itself chiefly as maintenance of the lobule-alveolar and duct systems in a condition very similar to that at parturition. Sufficient secretion was present to indicate partial retention of secretory activity. The degree of retardation appeared to be independent of the dose within the range used; and ovariectomy was without influence upon the regression in the treated animals.

These observations considered with previous data (5) are evidence that suckling prevents mammary regression and maintains lactation in mice by virtue of stimulation of the nipples eliciting release of lactogenic hormone which, in turn, maintains the mammary glands.

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INFLUENCE OF THE THYROID ON THE RESORPTION OF GONADOTROPIC HORMONES

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STUDIES (1–4) have shown that thyroidectomy in immature female rats will increase the ovarian response to injections of hypophyseal gonadotropic extracts but not to the injection of pregnancy serum extracts. Fluhmann (3) found that thyroid feeding decreased the gonadotropic effect of both types of preparations (pituitary or placental) and suggested, since the latter was free of thyrotropic principle while the former contained thyrotropic principle, that an induced condition of hyperthyroidism was produced by the injection of pituitary extracts in normal rats, this condition being responsible for the lessened response. The pregnancy extract not containing the thyrotropic factor was equally effective in normal and thyroidectomized animals.

Leonard (5), however, found that pituitary and urinary (castrate and testes tumor) gonadotropic extracts free from thyrotropic hormone were more effective in the absence of the thyroid and Leonard, Hisaw and Fevold (6) observed that the addition of thyrotropic hormone to pregnancy urine did not enhance the ovarian response. Leonard attempted to reconcile the existing experimental data by assuming that the presence of the thyroid inhibited or prevented the action of the follicle stimulating hormone but not the action of the luteinizing hormone. Tyndale and Levin (7) found that menopause gonadotropic extracts produced an enhanced effect in hypophysectomized rats, which effect was abolished by thyroid feeding. Leonard and De Frates (8) were unable to duplicate these results in the case of hypophysectomized rats, but did find that one of two preparations of hormone was more effective in thyroidectomized rats.

Hammett (9) made a very exhaustive study of the influence of thyroidectomy upon the reproductive activity of the rat. He found that when the thyroid was removed from young rats 45 to 56 days of age there was no apparent inhibition of function of the internal secretion of the gonads. He concluded that disturbances in reproductive ability were not due to any particular effect upon the endocrine function of the glands, but rather the result of the lowering of the anabolic level of the organism. Evans and Long (10) had previously shown that there was no change in the onset of maturity or the estrous cycle after thyroidectomy. Hammett found that in rats thyroidectomized at 100 days of age the ovaries not only stop growing but actually retrogress (11). The results following a longer interval after thyroidectomy do not, therefore, fit in with those cited in the first paragraph of this paper, which would predict greater rather than less ovarian function, if the response to the endogenous hormone would be the same as the response to injected material.

An examination of the differences in ovarian weights produced by thyroidectomy or thyroid feeding as compared with controls in any of the citations given above, will reveal that such differences are not greater in fact usually much less than are readily produced by divided dosage or delayed resorption (insoluble compound) of the pituitary hormone prep-

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[1] Resident in Surgery 1939–1940.

aration as compared with single dosage of the untreated extract (12). Since thyroidectomy or thyroid feeding produce well known changes in the rate of exchange of body fluids as indicated by heart weight changes, it appeared timely to consider the whole problem from the purely mechanical standpoint of release of hormone from the injection depot and rate of absorption of hormone by the ovary.

In this paper a comparison of the action of prolan and the pituitary gonadotropic hormone in normal and thyroidectomized immature rats was made, confirming earlier work. These experiments served as a basis for study of the action of the delayed resorption of the pituitary hormone under exactly the same experimental conditions. Zinc and copper, producing varying degrees of delayed resorption, were the agents used. In addition to these experiments the influence of thyroxin in decreasing the gonatropic effect of the pituitary preparation was confirmed and an experiment was included in which thyroxin administration abolished the effect of thyroidectomy.

EXPERIMENTAL

In each experiment, groups of 6 animals were used and a comparison was made between litter mates. The age at which the surgical operation was performed, the age of initial dosage, and the period of dosage are given in the tables, or under the headings covering the specific experiments.

The pituitary gonadotropic hormone and the prolan were the same preparations described in earlier publications (13, 14, 15). The zinc and copper combinations of the pituitary hormone were made according to the specific directions previously described (15, 16).

Squibb's crystalline thyroxin was used.

Heart weight changes served as an objective measure to determine the influence of thyroidectomy on body fluid exchange.

Technic of thyroidectomy. A mid-line incision about one-half inch in length is made midway between the sternum and widest portion of the mandible. The salivary glands and subcutaneous fascia are retracted laterally and the prethyroid muscles are separated in the mid-line exposing the trachea and thyroid gland. Exposure is maintained by small retractors holding these muscles laterally. The isthmus of the gland is then picked away by a forceps and then each lobe in turn is dissected using care to avoid injury to the structure in the carotid sheath laterally and the recurrent laryngeal nerve, which lies just deep to the gland on the longus colli muscle and somewhat under the convex lateral border of the trachea. When the lobe is being freed from the superior pole one must be certain it is free behind or the recurrent nerve may be injured by undue traction. There is usually a moderate amount of hemmorhage from the superior pole which is easily controlled by gentle pressure. Closure is effected by one suture in the skin.

If the recurrent nerve is torn, the animal shows a characteristic crowing inspiration associated with marked retraction of the thorax and cyanosis. This is readily alleviated by tracheotomy and leaving the incision open. Within 24 hours the tracheotomy is closed and the vocal cord has regained its tone sufficiently to allow a free passage of air.

Other, but less frequent, complications are: injury to the treachea when the isthmus is removed; secondary hemmorhage which may simulate injury to recurrent nerve and rarely sudden death probably from injury to the vagus nerve laterally.

Influence of thyroidectomy on the pituitary hormone. In experiment 1, 2, and 4, table 1, the rats were thyroidectomized at the age of 21 days. Dosage of the hormone was begun at the age of 23 or 24 days and was made once a day for 4 days. The rats were killed at the age of 27 to 28 days. In experiment 3, thyroidectomy was performed

the 23rd day of life and dosage was begun the same day. The rats were killed at the age of 27 days.

In experiment 1, 2, and 4, the heart weights of the thyroidectomized rats are 19 to 24% less than those of their respective controls. On a body weight basis the difference is 10 to 13%. In experiment 3 in which thyroidectomy was performed the day of initial dosage, there is no significant difference in heart weight on a body weight basis. There is a 10% absolute difference.

In experiment 1, there is a highly significant difference in the ovarian weights of control and thyroidectomized rats, confirming the observation of all previous workers. In experiment 2 in which the resorption of the hormone is delayed by zinc there is also a significant difference. In experiment 3 in which thyroidectomy was performed

TABLE 1. COMPARISON OF INFLUENCE OF DELAYED RESORPTION OF PITUITARY HORMONE IN NORMAL AND THYROIDECTOMIZED IMMATURE FEMALE RATS

Experiment	State of rats	Material injected in total dose per rat	Average wt. of ovaries	Average wt. of heart	Average body wt.
			mg.	mg.	gm.
1	Control	1 mg. untreated hormone	16± 1.0	240± 6	49±1
	Thyroidectomized	1 mg. untreated hormone	28± 2.0	183± 6	43±2.5
2	Control	1 mg. hormone+0.5 mg. Zn	52± 7	223±12	45±3
	Thyroidectomized	1 mg. hormone+0.5 mg. Zn	83±11	180±10	40±1
3	Control	1 mg. hormone+0.5 mg. Zn	52± 8	242± 9	46±1
	Thyroidectomized	1 mg. hormone+0.5 mg. Zn	67±10	219± 9	42±0.5
4	Control	0.5 mg. hormone+0.5 mg. Cu	44± 6	218± 7	49±3
	Thyroidectomized	0.5 mg. hormone+0.5 mg. Cu	45± 6	168± 6	43±3

the day of initial dosage there is no significant difference in ovarian weight. In experiment 4 in which Cu was used to delay resorption there is no difference in ovarian weights.

It will be noted that the 1 mg. of untreated hormone in normal rats produced a 16 mg. ovarian average which is a 33% increase over the control. The same amount of hormone combined with 0.5 mg. Zn produced an ovarian weight average of 52 mg., which is a 330% increase over the control. We have previously shown (12) that the same absolute increase may be produced by administering the untreated hormone in the same total dose in 5 injections per day over a 9-hour period. The same amount of hormone combined with 0.5 mg. Cu will produce an even greater increase in ovarian weight (about 700%) (12). In experiment 4, the maximum effect for a given amount of hormone has been produced by combining half the amount of hormone[2] used in the other experiments with 0.5 mg. Cu per rat. Under conditions which produce maximum stimulation for a given hormone dose in normal animals, thyroidectomy has no influence.

Influence of thyroidectomy upon prolan. In the one experiment performed, the amount of prolan which produced 34 mg. ovaries in controls, produced 39 mg. ovaries in animals thyroidectomized 3 days before dosage was begun. While the pituitary hormone is greatly influenced by divided dosage or delayed resorption, prolan is not greatly influenced. We have previously reported (17) upon the influence of a single dose versus the same total amount given once daily on 4 successive days. Data on the

[2] One mg. hormone augmented with Cu produces 100 mg. ovarian weight. It was not known whether this weight might readily be further increased, so half the dose was used.

TABLE 2. COMPARISON OF THE ACTION OF PROLAN IN NORMAL AND THYROIDECTOMIZED IMMATURE FEMALE RATS

Experiment	State of rats	Total dose of prolan per rat	Ovarian weight average	Heart weight average	Body weight average
			mg.	*mg.*	*gm.*
5	Controls	0.5 mg.	34±3.7	265±5	52±1
	Thyroidectomized	0.5 mg.	39±2.1	196±8	45±3

influence of daily divided dosage over a 4-day period were not forthcoming, so the following experiment was included.

Influence of divided dosage of prolan. Six immature rats were dosed once daily for 4 days with a total dose per rat of 12.5 U of prolan. Six litter mates received the same total dose administered at 9:00 A.M., 11:00 A.M., 1:00 P.M., 3:00 P.M., and 5:00 P.M., daily over the same 4-day period. The ovaries of the single daily dosed rats weighed 37±1.8; those that received the divided dosage weighed 48±3.0 mg.

Assuming on the basis of the experiments with the pituitary hormone, that the effect of thyroidectomy is adequately explained on the basis of delayed resorption, the results of the present prolan experiment are exactly what would be expected.

Influence of thyroxin dosage. Dosage of thyroxin, begun at the age of 18 days and continued through the 4-day period during which gonadotropic hormone was administered, produced a 23% increase in heart weight and significantly decreased the gonadotropic effect of the pituitary hormone. This latter observation confirms the original work of Fluhmann. See experiment 6, table 3.

TABLE 3. INFLUENCE OF THYROXIN ADMINISTRATION UPON THE RESPONSE TO PITUITARY GONADOTROPIC EXTRACT

Experiment	State of rats	Period of dosage of thyroxin, 0.2 mg./day/rat	Gonadotropic extract in total dose per rat	Average wt. of ovaries	Average wt. of heart	Average body weight
		age in days		*mg.*	*mg.*	*gm.*
6	Normal	—	1 mg. hormone+0.5 mg. Zn	62±11	257±16	49±5
	Normal	18–26	1 mg. hormone+0.5 mg. Zn	31± 5	318±11	43±3
7	Normal	—	1 mg. hormone+0.5 mg. Zn	65± 8	257± 8	54±1
	Thyroidectomized	23–26	1 mg. hormone+0.5 mg. Zn	52± 7	320±21	47±1

In experiment 7, table 3, thyroxin was administered to thyroidectomized rats the day of thyroidectomy. The influence of thyroidectomy in augmenting the gonadotropic response as illustrated in experiment 2, table 1, was abolished. The heart weight decrease was replaced by an increase.

DISCUSSION

These studies show that for the pituitary hormone thyroidectomy performed the day of initial dosage is less effective in augmenting the gonadotropic responses than when performed 2 to 3 days before initial dosage;[3] moreover, thyroxin dosage is able to counteract the influence of thyroidectomy. The influence of the thyroid would therefore be relegated to the influence of its secretion (thyroxin-like effect) and not to

[3] The 2 to 3-day interval was used to make our data comparable with earlier work. It is possible that a longer interval, depleting the body completely of endogenous thyroid hormone, would produce a greater effect.

any selective destructive effect on injected material. These studies also show that when the pituitary hormone is administered in such a way (delayed resorption) that its maximum gonadotropic effect is attained, thyroidectomy is without influence. The influence of thyroidectomy in augmenting the effect produced by a single daily injection of the soluble pituitary hormone is much less than the effect produced by injecting the same total dose of hormone 5 times per day over a 8-hour period. Thyroidectomy also augments the effect of the pituitary hormone given in zinc combination (equivalent to 5 daily injections over 8 hours). This augmentation is also less than is produced by administration of the hormone in copper combination. Since the influence of thyroidectomy is considerably less than the influence of prolonged delayed resorption, it is quite reasonable to assume that the influence of thyroidectomy is solely one of delayed resorption brought about by the decrease in rate of exchange of the body fluids following the absence of the thyroid hormone. This reasoning is strengthened by the correlation that response to prolan is not greatly influenced by delayed resorption nor by thyroidectomy, while response to the pituitary hormone is influenced by both delayed resorption and thyroidectomy.

SUMMARY

The influence of thyroidectomy in augmenting the gonadotropic response to the pituitary hormone given in single daily doses, was considerably less than the augmentation produced by divided dosage or delayed resorption (insoluble zinc or copper combination) of the same amount of hormone in the normal rat.

Under conditions (dosage as insoluble copper combination) eliciting maximum gonadotropic response for a given amount of pituitary hormone, thyroidectomy was without influence.

Thyroxin administration counteracted the effect of thyroidectomy under conditions in which thyroidectomy augmented the response to the pituitary gonadotropic hormone.

The response to prolan was not greatly influenced by divided dosage or thyroidectomy, in contrast to the response to the pituitary hormone, which response was markedly influenced by divided dosage or thyroidectomy.

It is concluded that when thyroidectomy exerts an augmentation effect, or thyroxin administration exerts an antagonistic effect, the phenomena may be explained by the decrease or increase in rate of exchange of body fluids which these procedures bring about, so decreasing or increasing the rate of hormone resorption.

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INHIBITION OF MAMMARY GROWTH BY LARGE AMOUNTS OF ESTROGEN[1]

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THE MAMMARY GLANDS of male or immature female animals of several species develop when estrogens and certain other sex hormones or chemically related substances are injected in adequate amounts (1). In some species the breasts may undergo complete development during periods of treatment with adequate amounts of estrogen alone, for example, guinea pigs (2) and monkeys (3). The addition of progesterone was required for complete mammary growth in other species (4). The glands of animals of the latter group developed only a system of mammary ducts when estrogens were injected; the development of the alveoli depended upon the administration of progesterone.

The administration of large amounts of estrogen to mice for long periods resulted in abnormal and stunted mammary glands (5). Similar observations have been made in rats (6). The restricted growth of the breast tissues of mice receiving large amounts of several estrogens was apparent within 15 days after the injections were started (7).

The inhibiting influence of large amounts of estrogen on the mammary glands of other species has not been investigated. An indication of the stunting of the breasts of monkeys was apparent in one report (3). The breast of one monkey given 0.8 mg. of estradiol benzoate was smaller than would have been expected in comparison to the other animals receiving smaller doses.

The influences of estrogens, injected in large amounts for prolonged periods, upon the mammary glands of mice, monkeys and dogs, is reported in the present paper.

MATERIALS

Mice from 7 different inbred strains varying in tendency to develop mammary tumors have been given variable doses of estrogens for periods of several months.[2] The mice of all strains have shown similar responses with respect to the extent of proliferation of mammary tissue. Therefore representative groups of mice from only one strain (C_3H) will be reported. Young male mice from 34 to 50 days of age were given weekly injections of 0.05 mg. estradiol dipropionate or 0.05 mg., 0.025 mg. or 0.01 mg. of estradiol benzoate.[3] Another group of mice was given 0.05 mg. of estradiol benzoate every second week (table 1). The hormones were dissolved in sesame oil in such concentrations that 0.05 cc. contained the amount administered at each injection. All injections were made subcutaneously. The injections were continued until tumors developed or until death occurred. The mammary glands were removed on the skin

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[2] The mice of C_3H, A, CBA, C_{57}, N, JK, $C_{12}I$ strains were obtained from stocks supplied by Dr. L. C. Strong.

[3] The estradiol benzoate used in these investigations was generously supplied by the Schering Corporation through the courtesy of Dr. E. Schwenk. The estradiol dipropionate was supplied by the Ciba Pharmaceutical Products, Inc. through the courtesy of Dr. E. Oppenheimer.

TABLE 1. THE RELATIVE GROWTH OF THE MAMMARY GLANDS OF MALE MICE OF THE C_3H STRAIN RECEIVING DIFFERENT LEVELS OF ESTROGENS

Amount of estrogen	No. of mice	Period treated days	State of mammary gland development I No.	II No.	III No.	No. of M. Ca [1]
0.05 mg. weekly DPB [2]	17	<300	15	2	—	1
		>300	—	—	—	
0.05 mg. weekly EB [3]	16	<300	11	1	—	5
		>300	1	3	—	
0.05 mg. EB every 2nd week	15	<300	—	3	2	12
		>300	—	7	3	
0.025 mg. EB weekly	12	<300	—	5	1	10
		>300	—	2	4	
0.010 mg. EB weekly	7	<300	—	1	3	4
		>300	—	1	2	

[1] M.Ca—mammary tumors.
[2] DPB—estradiol dipropionate.
[3] EB—estradiol benzoate.

and prepared as whole mounts according to methods described elsewhere (8). The animals were fed a prepared diet (Purina Fox Chow). The method of comparing the relative extent of development of the mammary glands will be presented later.

Six immature or young mature female monkeys (*Macacus rhesus*) and one young male monkey were used in the present experiments. Estradiol benzoate or dipropionate were injected subcutaneously into the legs twice weekly. The weekly doses varied from 0.2 mg. to 6.0 mg. (table 2). These animals were maintained on a mixed diet of fruit, vegetables and dog biscuit. Control glands were removed from 4 animals prior to starting injections. In addition the measurements of the glands of monkeys reported previously were included in one chart (chart 1) to present the response to smaller doses of estrogen. The glands, removed on the skin, were fixed after being flattened on cork boards and were later dissected and stained.

The mammary glands of 5 female fox terriers were studied. These dogs were from 2 litters and were 76 to 81 days of age at the time the injections were started. Two

TABLE 2. THE RESPONSE OF THE MAMARY GLANDS OF MONKEYS RECEIVING LARGE AMOUNTS OF ESTROGENS OVER PROLONGED PERIODS

No. of animal and sex	Wt. at start	Wt. at end	Period treated	Amt. estrogen [1]	Measurement of glands	Control
	gm.	*gm.*	*wk.*	*mg. wkly*	*mm.*	*mm.*
116♀	3207	3041	36	1.8	39×27	18×18
121♀	3321	2952	35	1.0	60×43	27×27
24♀	3410	2828	48	2.0	73×52	
175♀	4275	2918	26	6.0	68×51	
73♀	3650	3511	34.5	0.4	81×54	36×29
75♀	3100	3002	34.5	0.2	88×54	36×29
804♂	2495	2342	31	1.0	34×31	

[1] Estradiol benzoate in all animals except 175 in which estradiol dipropionate was used.

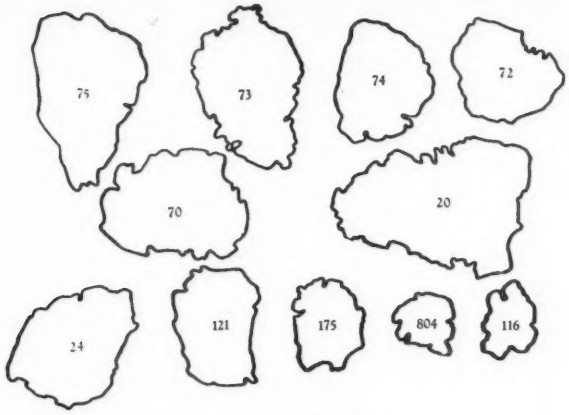

Chart 1. TRACINGS OF THE MAMMARY GLANDS OF MONKEYS RECEIVING VARYING DOSES OF ESTROGENS TO SHOW SIZE AND SHAPE OF THE GLANDS. The level of injection for monkeys 73, 75, 24, 121, 175, 804 and 116 are stated in table 2. The glands of monkeys 70, 72, and 74 were previously illustrated (3). The treatment of these animals was as follows.

Number and sex	Weekly dose estradiol benzoate	Duration of treatment	Weight	Size of glands
	mg.	*weeks*	*gm.*	
70♀	0.2	9	3320	71×52
72♂	0.4	22	3165	54×48
74♀	0.4 0.8	9 12.5	3320	61×47

The gland 20 was from an adult female weighing 4,700 gm. A photograph of 116 is shown in fig. 6.

dogs received 0.133 mg. and two, 0.333 mg. of estradiol benzoate weekly for variable periods (table 3). Later the doses were increased for variable periods as indicated in the table. One dog was given only weekly injections of sesame oil, the solvent for the estrogens used in the other 4 animals. All the dogs were fed fresh beef, a prepared dog food and evaporated milk. Gross amounts of the mammary glands were prepared by a method similar to that used for the glands of the monkeys.

OBSERVATIONS

The responses of the mammary glands of mice receiving estrogens have been

TABLE 3. THE RESPONSE OF THE MAMMARY GLANDS OF DOGS RECEIVING LARGE AMOUNTS OF ESTROGEN OVER PROLONGED PERIODS

No. of animal	Age started	Period treated	Amt. weekly	Mam. growth
	days	*wk.*	*mg.*	
1	76	82	0.333×81 wk. 7.0×1 wk.	none
2	81	72	0.133×67 wk. 7.0×5 wk.	slight
3	76	83	—	marked
4	81	67	0.333×67 wk.	slight
5	81	109	0.133×21 wk. 0.533×77 wk. 7.0×11 wk.	slight

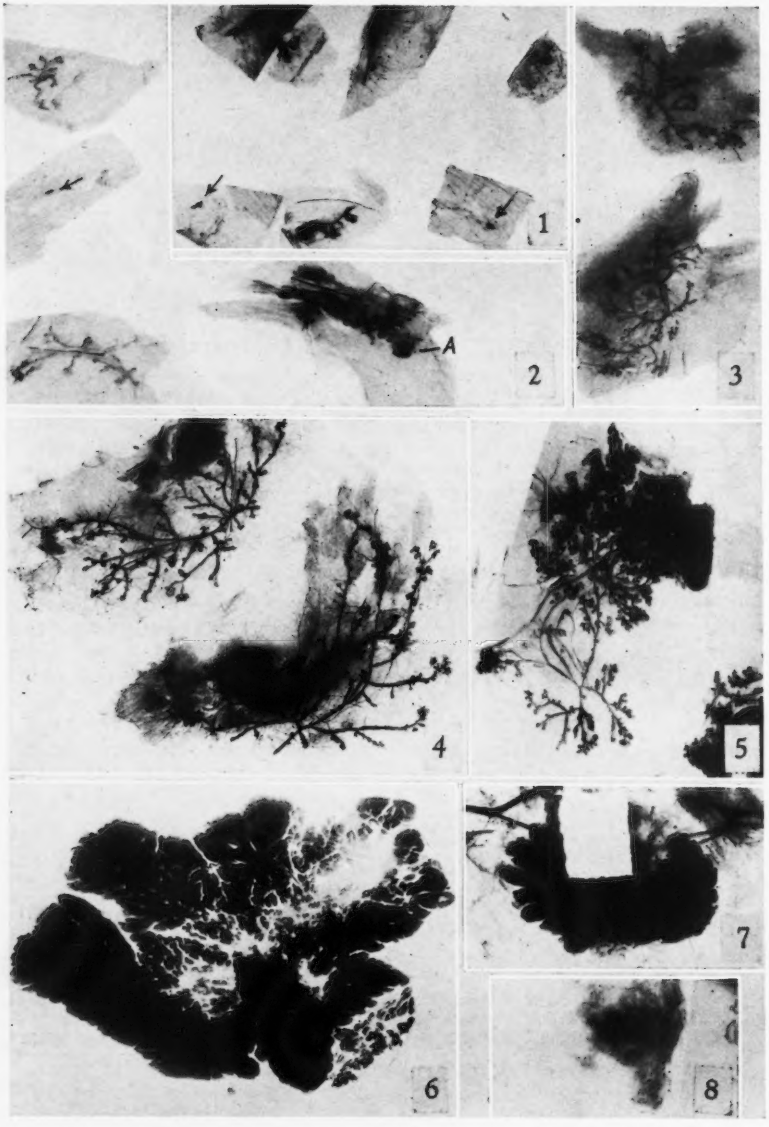

Fig. 1. Seven rudimentary mammary glands of a male mouse which had received weekly injections of 0.05 mg. estradiol dipropionate for 239 days starting at 36 days. Arrows indicate the smaller rudiments which are no longer than those of untreated mice. ×3. Fig. 2. Four mammary glands of a mouse which had received 0.05 mg. estradiol benzoate for 211 days. Three glands were slightly enlarged and one extremely small. One gland had small lobules of alveoli (A). ×3. Fig. 3. Two mammary glands (rated II) from a mouse which had received 0.05 mg. estradiol benzoate. ×3. Fig. 4. Two mammary glands (rated III) from a male mouse which had received weekly 0.01 mg. estradiol benzoate for 274 days. ×3. Fig. 5. One mammary gland (rated III) from a male mouse which had received weekly 0.025 mg. estradiol benzoate for 239 days. ×3. Fig. 6. A breast from

studied extensively under various experimental conditions. Most of these experiments have been of short duration and qualitative rather than quantitative responses have been considered most extensively. The quantitative nature of the responses to be described require that the usual responses of the glands be kept in mind.

The mammary rudiments of male mice persist as small ducts in the subcutaneous tissues. Nipples are not present. The rudimentary glands consist only of one major duct and one or more small branch ducts, the entire glands measuring not more than 1 to 3 mm. in any direction. The glands of male mice bearing ovarian grafts or receiving small amounts of estrogen attain a size and structure similar to that of mature virgin females. All of the rudimentary glands in the same animal, however, may not possess equal potentialities for growth. Some glandular rudiments may remain extremely undeveloped while others in the same animal respond to estrogenic stimulation by maximal growth.

Individual variations in the response of the glands of different animals and of different rudimentary glands in the same animal add to the difficulties of presenting quantitative responses. In order to quantitate the relative growth of the glands of mice to the different levels of estrogen the glands of 3 different mice were selected which showed slight, moderate and extensive development. The glands of the mice studied were then compared with these selected specimens and classified according to the 3 different extents of development (fig. 1–5). The largest glands of each mouse, with certain exceptions to be indicated later, were the ones used for comparison.

The glands of mice receiving weekly injections of 0.05 mg. of estradiol dipropionate or 0.05 mg. of estradiol benzoate for prolonged periods showed the least response (table 1; fig. 1, 2). Only 6 of 33 mice had glands rated II. The glands rated I showed either no appreciable response (fig. 1) or a very slight increase in size of the rudiments (fig. 2). In many animals only a few of the rudiments show appreciable evidence for growth during the entire course of treatment. Other rudimentary glands apparently failed to enlarge during the periods of treatment. They resembled those of untreated control animals of the same strains.

Not only was the growth of the glands greatly limited but many of the mammary rudiments which showed a slight proliferation presented evidence of an abnormal type of growth. Small nodular areas of alveoli appeared at one or more points along the small system of branching ducts (fig. 2). More rarely, from one point on one or more of the small glands, a diffuse overgrowth occurred greatly enlarging the area of the gland. These small nodular overgrowths as well as the more extensive proliferations arising from localized points are especially characteristic of the mammary glands of those strains in which the females develop mammary cancer. They have not been observed in the glands of mice from strains of low susceptibility to mammary tumors.

The mammary glands of mice receiving the estradiol dipropionate showed the greatest inhibition. None of these mice tolerated treatment for over 300 days. The average period of treatment was 213 days. The two mice in this group that had moderately developed glands were treated for 213 and 204 days. The duration of treatment for the entire group ranged from 137 to 299 days. The extent of mammary growth was not associated with the duration of the experiment. The mice receiving the estradiol benzoate survived an average of 265 days, a range of 81 to 368 days.

monkey 116 (TABLE 2) WHICH HAD RECEIVED 1.8 MG. ESTRADIOL BENZOATE WEEKLY IN 2 INJECTIONS FOR 36 WEEKS. This gland though small had completely developed lobules of alveoli. ×1.66. Fig. 7. A MAMMARY GLAND FROM A FEMALE DOG (4, TABLE 3). The clear square indicates a region removed for histological study. This dog had received 0.333 mg. estradiol benzoate weekly for 67 weeks. ×1.66. Fig. 8. AN EXTREMELY RUDIMENTARY GLAND OF A FEMALE DOG (1, TABLE 3) WHICH HAD RECEIVED 0.333 MG. OF ESTRADIOL BENZOATE WEEKLY FOR 81 WEEKS AND LATER A LARGER DOSE FOR 9 DAYS. The gland barely extended beyond the base of the nipples. ×1.66.

Three of the 4 mice in this group which showed the greatest mammary growth survived for over 300 days. Only 6 of the 33 mice of these two groups developed mammary tumors though the multiparous female mice of this strain show an incidence of such tumors of 80% or more.

None of the mice receiving 50γ of estradiol benzoate every second week, or 25 or 10γ weekly, had mammary glands rated less than II (table 1). Over one-half of the mice which had received the smallest dose had glands rated III (fig. 4). Such glands consisted of a branching system of ducts attaining dimensions of 1.5 cm. or more. Small nodular or larger and more diffuse overgrowths of glandular tissue were commonly observed in these groups as well as in the groups receiving the larger doses (fig. 5).

The average survival periods of the mice in these 3 groups were 325 ,265 and 275 days respectively. The incidence of mammary tumors in the mice of these groups ranged from slightly over 50 to 90% and was highest in the group receiving weekly doses of 25γ of estradiol benzoate weekly.

The glands of the mice in the last 3 groups also showed little indication that the extent of growth was related to the duration of treatment. The glands rated II were not as large as those of animals treated with optimal amounts of estrogen for periods of 15 days (7). Therefore the duration of the experiments, if the stimulation were optimal, could be expected to be exceeded in all of the animals, since the shortest experimental period was 81 days. The growth of the glands was probably not continuous throughout the entire experimental period, even though the glands did not attain their expected size, since a definite relation of the duration of the experiment and the size of the glands was not apparent.

The mammary glands of monkeys receiving the smallest dose of estradiol benzoate 0.200 and 0.400 mg. weekly for 34.5 weeks, showed the greatest area (chart 1, table 2). These glands (*monkey* 73, 75) were fully developed. The parenchymal tissue extended over an area of 81×54 and 88×54 mm. (greatest dimensions of the glands) and contained a normal lobular arrangement and extensive alveolar development. One male (*804*) and one female (*121*) monkey received weekly injections of 1.0 mg. of estradiol benzoate. Their mammary glands measured 34×31 mm. and 60×43 mm. after periods of treatment of 31 and 35 weeks respectively. The mammary glands of these 2 monkeys were also morphologically complete in that they consisted of a fully developed group of compound tubulo-alveolar glands.

The mammary gland of one female monkey, which received 1.8 mg. of estradiol benzoate weekly for 36 weeks, measured 39×27 mm. (fig. 6). The glands of another female monkey measured 71×51 mm. after 2.0 mg. of estradiol benzoate had been injected weekly for 48 weeks. No control gland had been removed from this monkey. This animal was apparently older than other monkeys of similar weight as the sexual skin did not show the usual prepubertal response and the glands were probably larger than those of the other female monkeys with the exception of *175* at the time injections were started.

The mammary glands of one monkey given weekly injections of 6 mg. of estradiol dipropionate for 26 weeks, measured 68×51 mm. As a control gland was not removed it is impossible to determine the extent of growth of the glands. As this was a large animal it is very probable that the glands were only very slightly enlarged during the course of treatment.

The dimensions of the glands as determined by the 2 greatest measurements at right angles to one another does not present a true picture of the size of the glands. Tracings of the fixed and stained glands were made on cellophane. From such tracings chart 1 was prepared. As few monkeys in the present series were given the

smallest doses the glands of 3 monkeys which have been previously illustrated (3) were included. The glands of 3 female monkeys (*116*, *175* and *121*) and of male monkey (*804*) were of similar size or much smaller than those of male *monkey 72* which had received 400γ of estradiol benzoate weekly for 22 weeks. The 4 former animals had received 1 to 6 mg. of estrogen weekly for 26 to 36 weeks. The glands of 2 of the above monkeys, *116* and *804*, were but slightly larger than those of one male monkey which had received 0.4 mg. of estradiol benzoate weekly for 9 weeks (*monkey 71*) (3).

The extent and shape of the fully developed mammary gland and the relation of the size of the gland to body weight have not been extensively studied. The glands have been observed to increase until monkeys attain a weight of 5 kg. (9).

The glands of adult female monkeys tend to be roughly triangular with the shorter side at the upper border (chart 1, *monkey 20*). The small glands of the monkeys receiving the large amounts of estrogen lack this triangular configuration. The stage of development and the uniformity of this shape of glands has not been determined sufficiently to enable the interpretation that the shape of the glands as well as their extent was altered in the present experiments.

The mammary glands of the young female dogs uniformly failed to show marked response to the injection of estrogens in amounts ranging from 0.133 mg. to 0.533 mg. weekly for 67 to 81 weeks. During the latter part of the experiment daily injections of 1 mg. were made for 9 to 77 days (table 3). The glands of one dog (1) were present only as rudiments after 82 weeks of continuous treatment. Only 3 of 4 glands removed from one side of the animal extended into the tissue below the base of the nipple. Its litter-mate sister (3) had very large and fully developed glands though it received no estrogenic hormone. This dog had gone through 2 estrous periods and was killed shortly after the end of the second prolonged metestrum. The mammary glands were thick and completely developed as might be expected during the latter part of pregnancy. The glands of the other 3 dogs showed slight growth (fig. 7, 8, chart 2). The glands of *dog 2* consisted largely of a branching network of ducts. In one area of an inguinal gland a small dense alveolar and a cystic area were present. Likewise the glands of *dog 4* and *5* contained cystic areas.

The individual glands of the dogs varied considerably in size in the control and in 3 of the injected animals (chart 2). The smallest gland of the control, however, was much larger than any of the glands of the treated animals.

Very little information is available on the influence of estrogen on the glands of dogs. Laqueur et al. (10) reported obtaining extensive growth of the glands of 1 young male dog by injections of 100 M.U. of estrone daily. No appreciable growth of the glands of 1 male dog was observed after the injection of 100 R.U. of estrone daily for 21 days (11). Houssay (12) stated that daily injections of 1000 to 10,000 I.U. of estradiol benzoate to male dogs for 40 to 90 days induced sufficient mammary growth to permit lactation following the injection of lactogenic hormone.

Until it can be demonstrated that small amounts of estrogen will induce mammary growth in dogs the inhibition observed in this series might be attributed to an inhibition of the ovaries. The information available at this time does not prove that the hormone of the corpus luteum is not essential for the development of the mammary glands of the dog. The development of large follicles and corpora lutea was prevented by the injection of estrogen. Whether the complete or marked inhibition of mammary growth of these dogs was due to an inhibition of the luteal function of the ovary or to the same factors as might be responsible for the relative mammary inhibition in male mice and monkeys can not be stated at this time.

The nipples of the dogs into which estrogen was injected underwent extreme

hypertrophy. They were uniformly larger than those of the control. It thus appears that quantitative or qualitative differences in response of the nipples and mammary glands may exist in dogs.

DISCUSSION

Estrogens have been reported to inhibit, at least certain of the activities of the hypophysis. The reciprocal hypophyseal-gonad relationship (13) was extended to explain the inhibitory effects of estrogen on lactation (14). Likewise estrogen inhibited body growth presumably by inhibiting the growth promoting principle of the pituitary (15). Mammary growth usually but not always failed to occur in hypophysectomized mice, rats and guinea pigs treated with estrogens (rev. 1, 17). Such observations lead to experiments showing a mammary growth factor in the hypophyses which has been considered independent of estrogens (17) and the conclusion that estrogens acted on the mammary glands indirectly through the pituitary.

Chart 2. TRACINGS OF THE MAMMARY GLANDS OF DOGS RECEIVING ESTROGEN (1, 2, 4, 5) IN VARIABLE AMOUNTS FOR PROLONGED PERIODS AND OF A CONTROL DOG (3), A LITTER MATE OF dog 1. Data pertaining to treatment are included in table 3. The largest and the smallest glands of the control dogs are demonstrated. All of the glands on the right side of dogs 1, 2 and 4, and the largest and smallest glands of dog 5 are presented. Figures 7 and 8 are photographs of glands of dogs 1 and 4 indicated by arrows.

The mammary glands of hypophysectomized rabbits, however, developed when estrogen was administered (16, 18), and the experiments with hypophysectomized rats have not been in complete agreement. The inhibitory effect of large amounts of estrogens on the mammary glands as shown by the present experiments could be explained by a decreased mammogenic activity of the pituitary. On the other hand, there is a lack of agreement on the necessity of the pituitary for mammary growth under all conditions. An explanation of the inhibitory effect of large amounts of estrogen on mammary growth based on the assumption that the mammary growth stimulating function of the pituitary is inhibited can not be made at this time.

The present experiments add further observations on the growth inhibiting effect of large amounts of estrogens in mice. The present experiments did not necessarily demonstrate that the rate of growth was slower in mice receiving massive doses. It seemed rather that mammary growth ceased shortly after the experiments started in those animals receiving the larger doses. The inhibitory effects of large amounts of estrogens on the mammary glands were apparent within 15 days after the first injection (7). If these large doses had been continued the effects would have persisted for over 200 days as shown in the present experiments.

The decreased extent of mammary growth also appears to be relatively greater

than the decreased increment of body growth in the treated dogs and mice. Mice 34 to 50 days of age at the time injections were started attained weights but slightly below that of the untreated controls. As determined by body weight, growth was largely inhibited in monkeys given large amounts of estrogen.

The marked failure of mammary growth in dogs receiving comparatively small amounts of estrogen was especially striking. As stated, it is not known at this time whether smaller amounts of estrogen would be effective. Dogs are uniquely sensitive to estrogen. One mg. per day was lethal when administered for sufficiently long periods, the dogs dying with an agranulocytosis and anemia as has been previously described (19). The smaller doses used in the early periods of the experiments resulted in almost a complete inhibition of hair growth (Gardner and Devita, unpublished).

The observations on breast growth in the monkeys reported here and previously indicate that weekly doses of 200 to 400γ of estradiol benzoate may give optimal proliferation and that weekly doses of 1 mg. or more inhibit the rate of growth and possibly reduce the ultimate size of the glands. The small glands developing in animals receiving large amounts of estrogen show complete formation of alveoli.

SUMMARY

The mammary glands of male mice given weekly injections of 50γ of estradiol benzoate and estradiol dipropionate were smaller than those of mice receiving smaller doses. The inhibition of mammary growth was nearly complete in mice receiving 50γ of estradiol dipropionate.

A similar inhibition of the mammary glands of immature or young female monkeys and one male monkey was noted when over 400γ of estradiol benzoate were injected weekly for prolonged periods.

The mammary glands of 4 young female dogs receiving weekly doses of 133 to 5333γ of estradiol benzoate for long periods and later, in 3 animals, larger doses for brief periods showed no or little growth. During the same period the mammary glands of a virgin control dog underwent extensive development.

In male mice of the C_3H strain, in which the females are susceptible to mammary tumors, the highest incidence of mammary tumors in the estrogen-treated male mice occurred in the group receiving doses permitting the more extensive mammary growth.

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THE QUESTION OF THYROID WEIGHT DURING PREGNANCY WITH FURTHER OBSERVATIONS OF ADRENAL WEIGHT IN LATE PREGNANCY

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IT IS WELL ESTABLISHED that the basal metabolic rate increases during pregnancy (1) and it may be assumed for this reason that the thyroids are especially active at that time. Histological examination indicates an hyperplasia with storing of colloid (2, 3). It is not unreasonable, therefore, to suspect that the thyroids increase in weight during pregnancy. At any rate, it is widely accepted that such is the case. Marine (4), in discussing the thyroid-gonad relationship, writes as follows: "This interrelationship has been recognized for centuries because of the enlargement of the thyroid during menstruation and pregnancy . . . ." This same idea of thyroid hypertrophy may be found in widely used textbooks of obstetrics (2). Although there are many reports in the literature regarding the microscopic structure and the gross size of the thyroid gland (5) during pregnancy, no quantitative data could be found on the thryroid weight–body weight ratio.

Since the animals had to be killed, to obtain the thyroids opportunity was offered to study further the weight of the adrenals in the different stages of pregnancy.

METHODS

Adult female guinea pigs were used, divided into 4 groups: non-pregnant, early pregnant, late pregnant, and early postpartum (72 hours or less). They were fed an adequate diet consisting of Rabbit Chow and cabbage. Food was withdrawn 30 hours before the animals were killed but water was left in the cage. The animal was weighed and then killed by a blow on the head; the large vessels in the neck were severed and after the animal was exsanguinated the thyroid and adrenals were removed. These organs were dissected free of extraneous tissue, washed momentarily in running water and blotted dry at once with filter paper. The organs were weighed in pairs to the nearest mg. The data in the accompanying table are expressed as the number of gm. of organ substance per kg. of body weight.

After the organs were weighed, the percentage of water was determined by placing them in a drying oven kept at a temperature of approximately 105° C. until a constant weight was reached.

The stage of pregnancy was determined as nearly as possible by inspection, palpation and weight determination. Animals included in the 'late pregnancy group' were practically at term when they were killed.

The body weight of the pregnant animals was corrected by deducting the weight of the uterus and contents and adding the normal average weight of uterus and adnexa predetermined on a large number of animals.

Postpartum animals were separated from their young as soon as the birth was discovered (within 8 hours).

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RESULTS

The data obtained from the thyroid are summarized in table 1. These show that there was no significant change in the thyroid weight–body weight ratio either in early or late pregnancy or during the postpartum period. This is in contrast with the data obtained from the adrenal gland given in table 2. These data show a noticeable increase in the adrenal weight–body weight ratio in late pregnancy and the postpartum period, but not in the early stage of pregnancy.

TABLE 1. THYROID WEIGHT–BODY WEIGHT (TW/BW) RATIOS DURING PREGNANCY AND PUERPERIUM

No. of animals	Condition	Average body wt.	TW/BW ratio	'p'[1]	Average % H_2O	'p'[1]
		gm.	gm./kg.			
30	Controls	486.2	0.1237	—	74.67	—
6	Early pregnancy	467.5[2]	0.1264	>1.0	76.66	0.4
12	Late pregnancy	575.9[2]	0.1199	0.9	75.26	0.9
11	Postpartum	550.1	0.1224	1.0	74.45	0.7

[1] 'p' refers to probability of difference from non-pregnant animals occurring by chance. A significant difference should have a 'p' less than 0.05 (Fisher) (10).
[2] Corrected for uterine contents.

The water percentage in the thyroid did not change significantly either during pregnancy or during the postpartum period. The adrenals, however, showed a significant increase in water percentage in the postpartum period.

TABLE 2. ADRENALS WEIGHT–BODY WEIGHT (AW/BW) RATIOS DURING PREGNANCY AND PUERPERIUM

No. of animals	Condition	Average body wt.	AW/BW ratio	'p'[1]	Average % H_2O	'p'[1]
		gm.	gm./kg.			
31	Controls	489.9	0.6176	—	68.2	—
6	Early pregnancy	498.0[2]	0.6948	0.3	70.43	0.1
12	Late pregnancy	575.9[2]	0.9137	<0.01	68.21	>1.0
11	Postpartum	550.1	0.9763	<0.01	69.97	0.05

[1,2] See footnote of table 1.

DISCUSSION

The results indicate that if there is increased thyroid activity during pregnancy and an hyperplasia, these changes must occur without an increase in the relative weight of the organ. It might be thought that the colloid storage and the increase in solid elements evidenced by the hyperplasia might be balanced by a decrease in water content. This hypothesis, however, is not in accord with our findings. As a matter of fact, the water content of the thyroid during the various stages of pregnancy as well as during the postpartum period, was remarkably constant. Since the size of the thyroid cannot be taken as a criterion of function, the lack of increased relative weight of the thyroid during pregnancy must not be interpreted as a indication of thyroid inactivity.

In contrast to these negative findings in the thyroid, a number of workers (3, 6, 7, 8) have found an increase in size and weight of the adrenals during pregnancy. None of them, however, paid especial attention to the stage of pregnancy. If no distinction is made between the ratios in early and late pregnancy, mean values for groups of pregnant animals may, however, show no difference from the normal due to the reduction of the mean by the unaltered ratios in animals in early pregnancy.

In these reported cases of adrenal weights in gravid animals, it must be assumed

that they were far advanced in pregnancy. Sleeth and Van Liere (9) have recently found that there is no significant change in the adrenal weight–body weight ratio in the guinea pig during pregnancy. The majority of their animals were in the early stages of pregnancy. Differences in reports in the literature may be reconciled by distinguishing between early and late pregnancy.

The work reported here shows clearly that in the late stage of pregnancy there is an increased weight of the adrenal, but not in the early stage, which confirms the previous report made by Sleeth and Van Liere (9).

It is of interest that in addition to the increased adrenal weight in late pregnancy over the normal, 48%, there is a further 10% increase in the postpartum period. This probably may be explained as being due in part at least, to an increase in water content brought about by the redistribution of water after parturition. The data show that there is a small but significant increase in water percentage in the adrenals in the postpartum period.

SUMMARY

There is a marked increase in the adrenal weight–body weight ratio in late pregnancy (48%) and in the postpartum period (58%), but there is no increase in early pregnancy. There is a slight but significant increase in water content of the adrenals in the postpartum period.

There is no increase in the thyroid weight–body weight ratio in the guinea pig in pregnancy, either early or late, or during the postpartum period, nor is there any significant change in the water content of the thyroid.

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THE RELATION OF THE THYROID TO MAMMARY GLAND GROWTH IN THE RAT

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THERE HAVE APPEARED in the literature several reports which indicate that the thyroid gland is not essential for mammary growth and function. Dragstedt, Sudan and Phillips (1) observed that in the complete absence of the thyroid and parathyroid glands, bitches become pregnant and lactate following parturition if tetany is controlled. Nelson and Tobin (2) thyroidectomized 2 groups of rats, one group between the 13th and 16th days of pregnancy and the other group prior to the incidence of pregnancy. The animals which survived the operation delivered normal litters and, in every instance, raised their young. Folley (3), however, reported subnormal lactation in rats thyroidectomized during pregnancy or lactation.

Of particular significance is the work of Nelson and Hickman (4) who reported marked mammary development following estrogen treatment in either thyroidectomized rats or thyroidectomized-castrated rats. Weichert, Boyd and Cohen (5) reported that induced hyperthyroidism in normal female rats resulted in pseudopregnancy and marked stimulation of the mammary glands, although typical lobule formation did not occur.

Of the many investigations reported, none was concerned with a study of the comparative development of the mammary gland in the thyroidectomized normal and castrated female rat and such animals treated with estrogen. The results on these problems are presented here.

METHODS

Albino or piebald female rats were subjected to thyroidectomy between the ages of 30 and 53 days. They were fed Purina dog chow ad libitum supplemented with lettuce. An estrogen (progynon-B) was used to stimulate growth of the mammary glands in the castrated rats. Body weighings were made before and at the termination of the experiments, and ovarian weights were recorded at autopsy. The mammary glands from each animal were stained and mounted in toto for comparative purposes. Examination for thyroid fragments was made under the binocular microscope at necropsy.

Effect of Thyroidectomy on Mammary Growth of Normal Female Rats

A group of 5 female rats, 50 days old, were thyroidectomized and 3 others of the same age served as controls. After a period of 54 days the rats were killed and obser-

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vations made as indicated above. The average gain in body weight of the thyroidectomized animals was 62 gm. as compared with 86 gm. for the controls. The average ovarian weight for the operated animals was 43.8 mg. as against 66.6 mg. for the controls.

The mammary glands of the thyroidectomized rats, on the whole, showed greater development of lateral and end buds than did those of the controls, (fig. 1, 2). An increased extension of the duct system did not always occur. The mammary glands, however, could not all be separated into 2 groups as far as the degree of development was concerned because one of each group could be interchanged. Further examination of these 2 cases revealed that the control rat had gained less in body weight (69 gm.) than any of the other controls while the thyroidectomized rat gained more (86 gm.) than any other in its group (compare with the averages given).

The experiment was repeated using 8 thyroidectomized and 7 control rats. The operation was performed on 45-day-old rats and they were killed 62 days later. The results were disappointing in that it was difficult to separate the glands of the 2 groups as in the previous experiments. However, in another group of rats of similar age, 9 were thyroidectomized and 8 were kept as controls at the same time and under the same conditions as above. These rats were injected with 2.5 R.U. of menopause urine extract for the last 5 days before killing. The mammary glands of 8 of the 9 thyroidectomized injected rats showed greater development of the lateral and end buds than did 7 of the 8 controls.

The gain in body weight, of that rat from each group which did not conform, differed sufficiently from the average of its group to warrant mentioning. The thyroidectomized rat gained 96 gm. in body weight (average of group 65 gm.) and the control rat gained 81 gm. (average of group 99 gm.).

Effect of Thyroidectomy on Mammary Growth of Castrated and Estrogen Treated Castrated Rats

The most striking results were obtained when estrogen was injected into a series of castrated thyroidectomized rats and into castrated controls. Nine 30-day-old female rats were thyroidectomized and at 49 days of age they were castrated along with 7 controls. After a period of 31 days, the rats of both series received 5 R.U. of progynon-B daily for 10 days and were then killed. The average gain in body weight of the thyroidectomized rats was 122 gm. as compared with 134 gm. for the controls. In all cases, a greater development of the mammary glands was observed in the thyroidectomized rats when compared with the controls. Figures 3 and 4 illustrate the degree of development of the lateral and end buds and to some extent the alveoli. The mammary glands of the castrated injected controls showed an extensive duct system with numerous lateral and end buds but not comparable to that of the operated animals.

These results led us to investigate the effect of thyroidectomy on the mammary glands of castrated female rats without the administration of estrogen. Four rats, 46 days old were castrated, 5 days later they were thyroidectomized and after 71 days they were autopsied together with 3 control rats which had been castrated only. The average gain in body weight of the thyroidectomized rats was 104 gm. as compared to 116 gm. for the controls. The mammary glands of all the castrated thyroidectomized rats were better developed than those of the controls (fig. 5, 6). This was characterized by an enlargement of the ducts and an increased number of lateral buds in the region of the teats while the control glands consisted mainly of a naked duct system. The experiment was repeated on 8 adult female rats which had been castrated when they were 31 days old. Four of these rats were deprived of their thyroids 44 days before autopsy. The mammary glands of 2 of the thyroidectomized

rats showed the characteristic enlargement and resembled those of the preceding experiment. However, the mammary development of the other 2 thyroidectomized rats resembled the controls and did not show the influence of thyroid removal. The gain in body weight of the latter animals was approximately equal to that of the controls

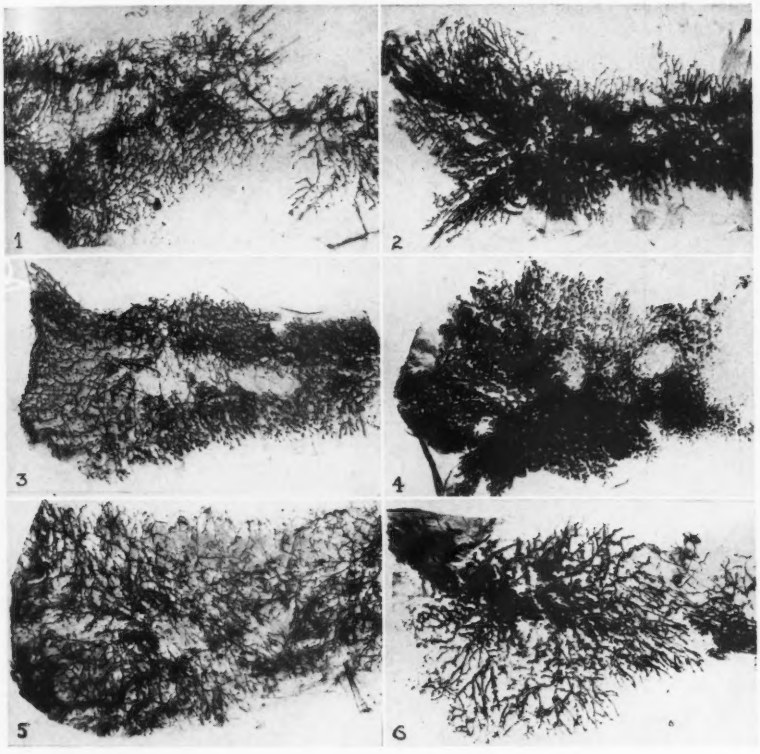

Fig. 1. MAMMARY GLAND OF NORMAL FEMALE RAT. Fig. 2. MAMMARY GLAND OF THRYOIDECTOMIZED FEMALE. Fig. 3. MAMMARY GLAND OF SPAYED FEMALE INJECTED WITH ESTROGEN. Fig. 4. MAMMARY GLAND OF SPAYED THYROIDECTOMIZED FEMALE INJECTED WITH ESTROGEN. Fig. 5. MAMMARY GLAND OF SPAYED FEMALE. Fig. 6. MAMMARY GLAND OF SPAYED THYROIDECTOMIZED FEMALE. (All magnifications ×1.6)

and was twice that of the thyroidectomized rats which had good mammary development.

In the final experiment an attempt was made to compare the mammary growth of thyroidectomized castrated rats injected with thyroxin and an estrogen with that of castrated controls injected only with estrogen. Six female rats were castrated at 46 days of age, thyroidectomized at 51 days, and at 113 days they were injected with 1/6 mg. of thyroxin plus 5 R.U. of progynon-B daily for 10 days. Seven control rats were castrated at 46 days of age and at 113 days they were injected with 5 R.U. of progynon-B daily for 10 days. At autopsy, the mammary glands of the 2 groups could be distinguished from each other only by a slightly greater development of the lateral buds in the older portions of the glands of the thyroidectomized animals. Otherwise, the glands were similar.

DISCUSSION

In view of the stimulating effects of thyroid treatment on mammary growth (5) the resulting increased mammary development after thyroidectomy was unexpected. In female rats, with only their thyroids removed, there was an increase in the degree of development of the mammary glands, although this did not consistently occur. Perhaps the presence of the ovaries (with their corpora lutea) masked to some extent, or modified, the effects of thyroid removal. The best results were obtained when both thyroids and ovaries were removed and estrogen was administered. Under these conditions, the thyroidectomized animals evidenced a markedly superior mammary growth. When menopause urine extract was given to thyroidectomized and normal females, the mammary development in the operated animals was distinctly superior.

That thyroxin is the active principle concerned in these experiments was observed when this hormone was injected into the thyroidectomized animals. The mammary glands of the thyroidectomized castrated rats treated with thyroxin and estrogen could be differentiated only with difficulty from those of normal castrated rats receiving only estrogen. A corrected dosage of thyroxin in such experiments as these would probably give better results.

This peculiar observation of either an excess or deficiency of thyroid substance favoring mammary development might not be so paradoxical, on further examination, as it first seems. The mechanism by which thyroxin favors mammary development may be different from that which explains in what manner a lack of thyroid also favors mammary development. In the first instance, hyperthyroidism (6, 7) increases the amount of estrogen required for the production of an estrous condition in castrated rats and even inhibits the activity of the follicle stimulating hormone (8, 9), which in turn would cause the formation of less estrogen. This would tend to make the estrogen stimultation of mammary growth less than normal. On the other hand, the functional corpora lutea which result from hyperthyroidism (10) would offset this by giving an estrogen + progesterone effect. Such combinations of hormones are known to produce lobule proliferation greater than that produced by estrogen alone (11). The net result would be a marked improvement in mammary growth.

In the hypothyroid state, it is possible that the sensitivity of the end organs to estrogens is increased, although there is no experimental evidence to suggest this other than that indicated here. It is known, however, that the effects of the follicle stimulating hormone are increased by thyroidectomy, producing larger ovaries and uteri than in the control animals (12). The larger uteri may be caused by the greater production of estrogen by the ovaries, by a more efficient utilization of the estrogen, or by both factors. Thyroidectomy in the rat does not seem to interfere a great deal with the reproductive cycle except to lengthen the period slightly (13), and hence this would lead one to believe that a slightly prolonged estrous cycle is not an important factor in the results.

A more efficient utilization of estrogen by the thyroidectomized rat may be an important factor in this work. While small doses of estrogen elicit only duct development of the mammary gland in the castrated rat (11, 14) larger amounts of estrogen stimulate varying degrees of alveolar development (11, 15, 16).

The apparent correlation between the degree of mammary development and gain in body weight, in those animals whose mammary glands did not attain the same degree of development as the majority in a given group, should be considered. It is known that thyroidectomy interfered with normal growth of the rat (7) yet it is seen in the operated group that the mammary glands attain a greater degree of development. If thyroid removal influences body growth rate by bringing about an inferior

nutritive state, one might expect to find the mammary glands at a low degree of development. Inanition has been shown by Astwood et al. (18) to influence mammary growth adversely. Apparently this is not the case here, because the thyroidectomized rats with the lower body growth rate possessed the best developed mammary glands and those which grew the most had mammary glands resembling the controls. It is remotely possible that the operated rats in our experiments which did not conform were incompletely thyroidectomized and not detected. On the other hand, the rats with intact thyroids and whose mammary glands resembled those of the operated rats might have had an abnormally functioning thyroid. It is difficult at present to explain these exceptional cases.

Astwood et al. (18) suggest that minor disturbances in the nutritional state, such as those brought about by partial adrenalectomy, caused injected estrogen to induce abnormal mammary growth. This was not observed in our experiments when the thyroids were removed. More recently, Butcher (19) has shown that adrenalectomy favors mammary development in either normal or castrated rats that were underfed. Thyroidectomy also favors a better mammary development in either castrated or estrogen injected castrated rats when compared with the appropriate controls. Since the rats in our experiments were not underfed, such differences in the state of development must be attributed to thyroid removal. Whether or not this is a direct effect remains to be proved.

SUMMARY

Thyroidectomy in young female rats resulted in changes in the mammary gland characterized by a thickening of the ducts and an increase in the number of lateral and end buds.

Thyroidectomy in spayed female rats brought about similar changes when compared with spayed controls.

Thyroidectomized spayed rats treated with estrogen showed the same effects as in the above instances, only to a greater extent, when compared to appropriate controls.

Thyroxin administered to thyroidectomized rats treated with estrogen tended to prevent these changes. A discussion of the possible mechanism is presented.

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STUDIES ON CORPUS LUTEUM METABOLISM

I. EFFECTS OF INTERMENSTRUAL ADMINISTRATION OF TESTOSTERONE PROPIONATE[1]

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IN AN EARLIER REPORT by some members of our group (1) upon androgenic metabolism in women, attention was called to the advisability of studying the effects of injections of testosterone propionate upon the normal ovarian function of healthy women. One of the hypotheses, upon which much of the use of androgens in gynecic therapy is founded, presupposes that these agents are capable of 'overriding' or 'negating' normal ovarian function. The present study is pertinent to this theory.

METHODS

A group of 11 young healthy women, whose examinations had indicated the non-existence of any significant endocrine disease was selected for this study. The common complaint of these patients was dysmenorrhea. Their ages ranged from 18 to 33 years; the average being 23.9 years.

Levels of ovarian function prior to therapy and during therapy were judged by the following studies: (*a*) determinations of daily urinary titers of sodium pregnandiol glucuronide by the method of Venning (2) and (*b*) estimations of daily urinary titers of 17-ketosteroids, using the extraction method of Cuyler and Baptist (3) and the colorimetric method of Oesting (4), were done during the intermenstrual phases of menstrual cycles; (*c*) the endometrial responses, which existed at the end of these cycles, were determined by studies of endometrial biopsies taken within the first 12 to 18 hours of bleeding.

Testosterone propionate[3] in oil was given intramuscularly in individual doses of 25 mg. at 2-day intervals during the first half of 15 menstrual cycles. The average time of initiation of therapy was the 10th day of the cycle and that of its completion was the 16th day of the cycle. During these studies a total of 1,625 mg. of testosterone propionate was given, the average dose per cycle being 108 mg.

DATA

Excretion of pregnandiol complex. The 129 determinations made during 8 pre-treatment cycles of 8 patients yielded a total recovery of 251 mg. of sodium pregnandiol glucuronide, the average total cyclic excretion per patient being 31.3 mg.

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[2] Ciba Fellow.
[3] Perandren, supplied by Ciba Pharmaceutical Products, Inc., and oreton, supplied by the Schering Corporation.

The 170 determinations made during 8 cycles of therapy given respectively to the 8 patients yielded a total recovery of 251 mg. of sodium pregnandiol glucuronide, the average total cyclic excretion per patient being 31.3 mg.

Excretion of 17-ketosteroids. The 110 determinations prior to therapy and 54 during therapy were made upon the urines of 7 patients. During therapy the average urinary titers of these patients were increased by amounts which varied from 1.5% to 65.4% of pretreatment values.

Endometrial responses. Endometrial responses prior to and during 10 cycles of therapy, determined in the cases of 7 patients, are summarized in table 1.

TABLE 1. ENDOMETRIAL RESPONSES TO ANDROGENIC THERAPY

Case number	Endometrium before treatment	Endometrium after treatment
1	Normal progestational	Normal progestational
2	Normal progestational	Normal progestational
3	Normal progestational	Normal progestational
4	Mixed progestational	Mixed progestational (during 2 cycles)
5	Mixed progestational	Mixed progestational
6	Persistent estrogenic	Persistent estrogenic (during 3 cycles)
7	Hypoestrogenic	Hypoestrogenic

DISCUSSION

These data do not indicate that the dosage levels of testosterone propionate employed produce any significant alterations in ovarian function in general and of corpus luteum function in particular. These observations do not permit the assumption that larger doses of androgens would act similarly.

A fortunate choice of patients with varying levels of ovarian function, as judged by endometriotropic responses, permits the following deductions. Normal ovarian function and varying grades of ovarian failure were not affected adversely by therapy; vice versa, there was observed no degree of recovery from diverse grades of ovarian failure, as a result of therapy. Various theories of synergism or augmentational effects of the various sterols have suggested the likelihood of this latter action.

What symptomatic effects androgenic therapy of this nature and amount produces, in such conditions as dysmenorrhea, cyclomastopathy, premenstrual tension, etc., cannot be explained upon the basis of any ovarian 'negating' or ovulational inhibiting action.

SUMMARY

Administration intramuscularly of testosterone propionate in 4 to 5 doses of 25 mg. each at 2-day intervals during the first half of 15 menstrual cycles of 11 healthy young women produced no significant alterations in pretreatment levels of ovarian function, as judged by the urinary excretion of the pregnandiol complex and by endometriotropic responses.

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STUDIES ON CORPUS LUTEUM METABOLISM

II. EFFECTS OF INTERMENSTRUAL ADMINISTRATION OF ESTROGENS[1]

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A NUMBER of workers, including Zondek (1) and Sturgis and Albright (2), has assumed that ovulation and corpus luteum activity are disturbed, and perhaps even inhibited, by intermenstrual administration of estrogens. The clinical use of these sterols in the treatment of dysmenorrhea and cyclomastopathy frequently is based upon such a theoretical modus operandi. Studies by some members of our group (3) have indicated that the concomitant administration of estrogens and progesterone results in more effective metabolism and utilization of progesterone than the use of progesterone alone and some evidence has been encountered that in various grades of ovarian failure estrogenic therapy apparently enhanced corpus luteum metabolism. The present study deals with the effects of intermenstrually administered estrogens on the corpus luteum function of normal, healthy women.

METHODS

A group of 13 essentially healthy, regularly bleeding women who presented no significant endocrine disease, was selected for the study. The complaints of these patients were as follows: dysmenorrhea, 11 patients; menstrual pruritus, 1 patient; pigmentation with menses, 1 patient. Their ages ranged from 20 to 42 years, the average being 27 years.

The methods of judging levels of ovarian function prior to and during therapy are described in the preceding report (4). These embraced studies of urinary titers of sodium pregnandiol glucuronide and those of endometrial specimens taken at the onset of episodes of bleeding.

A total of 17 menstrual cycles was studied during which estrogenic substances given parenterally and orally were employed either alone or in combination. The substances used and details as to dosages are as follows.

Oral estrogenic substance. Estriol glucuronide[3] was used in 11 of the 17 cycles studied; alone in 7 cycles, and in combination in 4 cycles. The daily dose ranged from 120 to 1800 O.U., the average being 665 O.U. A total of 114,360 O.U. was given, an average of 10,396 O.U. per cycle. The average time of initiation of therapy with estriol glucuronide was the 6th day of the cycle and that of its completion was the 23rd day of the cycle.

Estrogenic substances in oil. Estrone[4] was given intramuscularly in individual

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[2] Ciba Fellow.
[3] Emmenin, supplied by Ayerst, McKenna & Harrison.
[4] Amniotin, supplied by E. R. Squibb & Son. Theelin, supplied by Parke, Davis & Company.

doses of 10,000 I.U. (in 3 cycles) or 20,000 I.U. (in 5 cycles) at 2-day intervals. during the first half of the menstrual cycle. It was used alone in 5 cycles, and in combination in 3 cycles. A total of 500,000 I.U. was given, an average of 62,500 I.U. The average time of initiation of therapy with estrone was the 10th day of the cycle and that of its completion was the 17th day of the cycle.

Estradiol dipropionate[5] was employed in the case of 1 patient during 2 successive cycles. It was given alone in the first cycle and in combination with estriol glucuronide in the second cycle. Individual doses of 15,000 R.U. were given at 2-day intervals. In each cycle a total of 75,000 R.U. was given, injections beginning on the 6th day of the cycle and ending on the 14th day of the cycle.

DATA

Excretion of pregnandiol complex. The 60 determinations made during 5 pretreatment cycles on 4 patients yielded a total recovery of 68 mg. of sodium pregnandiol glucuronide, the average total cyclic excretion per patient being 13.6 mg. The average value per determination was 1.1 mg.

The 44 determinations made during 5 cycles of therapy given respectively to the 4 patients yielded a total of 89 mg. of sodium pregnandiol glucuronide, the total cyclic excretion per patient being 17.8 mg. The average value per determination was 2 mg.

Endometrial responses. Endometrial responses prior to and during 12 cycles of therapy, determined in the cases of 9 patients, are summarized in table 1.

TABLE 1. ENDOMETRIAL RESPONSES TO ESTROGENIC THERAPY

Case number	Endometrium before treatment	Treatment	Endometrium after treatment
1	Normal progestational	Es[1]	Normal progestational
2	Mixed progestational	E→Es	Normal progestational
3	Mixed progestational	E	Mixed progestational
4	Mixed progestational	E	Mixed progestational
		Es	Mixed progestational
5	Mixed progestational	E+Es→Es	Mixed progestational
		E+Es→Es	Mixed progestational
6	Mixed progestational	EdD	Mixed progestational
		EdD+Es→Es	Mixed progestational
7	Persistent estrogenic	Es	Mixed progestational
8	Persistent estrogenic	Es	Mixed progestational
9	Persistent estrogenic	E	Mixed progestational

[1] Es, Estriol glucuronide; E, Estrone; EdD, Estradiol dipropionate; E+Es→Es=Estrone and estriol glucuronide given concurrently during the first half of the cycle followed by estriol glucuronide during the last half of the cycle.

DISCUSSION

These data indicate that estrogens given in the manner and amounts employed in these studies exert no depressing effects upon corpus luteum function. In fact, evidence is submitted, that in some instances, at least, an augmentation or stimulation of this function occurred.

The evidence in favor of the view that estrogens may increase corpus luteum function may be summarized as follows. Endometriotropic findings include the progression during therapy of a mixed progestational endometrium to a normal progestational one and alteration of the persistent estrogenic endometria of 3 patients into

[5] Diovocylin, supplied by Ciba Pharmaceutical Products, Inc.

mixed progestational ones. Data based on urinary titers of pregnandiol complex are significant: the average total cyclic excretion of this compound per patient was increased 30.9% during therapy; the average recovery per determination was increased 83.6% during therapy. These facts justify our skepticism of the 'ovulation inhibiting' or 'corpus luteum negating' theory, which was aroused by the occasional occurrence of pregnancy during this form of therapy for dysmenorrhea.

Most of the dosages employed in these studies are somewhat smaller than those employed in the studies of Sturgis and Albright (2) but they are, with few exceptions, much larger than those commonly reported effective in the treatment of dysmenorrhea. These workers reported that 9 patients, who had received therapy embracing intermenstrual injections of 10,000 R.U. of estradiol benzoate every 3 days for 3 to 14 doses, showed estrogenic endometria at the conclusion of treatment, whereas during the cycles prior to therapy progestational endometria had been encountered. Patient 6 of our studies, who during one cycle received estradiol dipropionate 15,000 R.U. every 2 days for 5 doses and who during the next successive cycle received similar therapy combined with a total of 19,800 O.U. of estriol glucuronide, compares favorably in the matter of dosage with any of the patients studied by Sturgis and Albright. This patient experienced no adverse effects as regards corpus luteum function.

These deductions seem warranted from our studies: (*a*) estrogens, when given in total cyclic doses as great as 75,000 R.U. and when therapy is begun as early as the 6th day of the cycle and concluded at or about the time of expected ovulation, fail to depress corpus luteum function; (*b*) estrogens, when given intramuscularly in these amounts and in smaller doses, may enhance corpus luteum function; (*c*) the clinical responses in dysmenorrhea and other conditions reported from this form of therapy are more likely due to the latter action; (*d*) if estrogens are capable of inhibiting ovulation, larger doses than those employed by us are necessary.

SUMMARY

Various intermenstrual dosages of diverse estrogens, as great as that of 15,000 R.U. every 2 days from the 6th to the 14th days of the menstrual cycle, given during 17 cycles of 13 healthy women failed to produce any significant depression of corpus luteum function. Studies of urinary titers of the pregnandiol complex and of endometriotropic responses, on the contrary, seemed to indicate the occasional occurrence of augmentation of the progestational phenomena.

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THE EFFECT OF EXPERIMENTALLY INDUCED PSEUDOPREGNANCY UPON THE SURVIVAL OF ADRENALECTOMIZED CATS[1]

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RECENTLY a great deal of attention has been directed toward the physiological similarity between the hormones of the adrenal cortex and the corpus luteum. The present study is concerned with the use of a menopause urine extract to induce estrus and mating in mature female cats, and with the effects of the resultant pseudopregnancy or pregnancy upon the life-span of adrenalectomized females.

Rogoff and Stewart (1, 2) brought attention to the fact that pregnant and pseudopregnant dogs could survive adrenalectomy for periods as long as 65 days without treatment of any sort. They suggested that the internal secretion of the corpus luteum might be responsible for the lengthened survival time. However, it was shown that pregnancy did not increase the life-span of the adrenalectomized cat (3, 4). Rogoff and Stewart (5) made similar observations for this animal.

More recently Swingle et al. (6, 7) were able to show that induced estrus and pseudopregnancy were as effective in prolonging the lives of adrenalectomized dogs as the naturally occurring luteal phase. Similarly, the pseudopregnant, adrenalectomized ferret lives longer than the control (8), and the lives of adrenalectomized rats are prolonged when functional corpora lutea are present in the ovaries (9). In addition, pure crystalline progesterone has been found effective in ameliorating or preventing the symptoms of adrenal insufficiency in rats (10, 11, 12), in ferrets (13), and in castrate female cats (14).

Because of the non-spontaneity of ovulation in the cat, pseudopregnancy or the postovulatory phase of the ovary and the uterus must be artificially induced. Sterile coitus (15, 16) and stimulation of the vagina and cervix of the estrous cat (17) have been found to result in a typical luteal phase. Foster and Hisaw (18) induced estrus and pseudopregnancy in the cat by using pure follicle stimulating and luteinizing hormones of the anterior pituitary. In this way they were able to study the hormonally induced luteal phase in detail.

METHODS

The efficacy of an extract of menopause and castrate urine, gamone,[3] in inducing a typical estrus and mating response was determined in a series of 22 adult cats. In addition 3 animals were killed as untreated, anestrous controls. In all cases the animals

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were killed and autopsied at the end of the experiment, and in crucial cases both ovaries and tissue from each uterine horn were removed and prepared for histological examination. During the period of hormone injection and the duration of estrus, the vaginal smear picture was followed daily.

Gamone was received in powder form and had been assayed at 5 R.U. per mg. It was dissolved in distilled water and injected intraperitoneally. Since this material had never been used on the cat, nothing was known about the dosage necessary to produce estrus or adequate follicle stimulation in this species. It had, however, been used in the dog (6, 7, 19) to produce estrus. Leathem (19) found it was necessary to inject follutein (Squibb pregnancy urine hormone), following gamone treatment, in order to obtain fertile ovulation.

In the present investigation, 9 of the gamone injected cats were employed in the studies on the effects of pseudopregnancy on the survival after bilateral adrenalectomy. The right adrenal gland was removed in each case under nembutal anesthesia, and during the interval between removal of right and left glands, the animals received injections of gamone required to produce estrus. Throughout the mating period each animal was allowed to mate as often as possible. Immediately at the close of estrus, the animal was placed under ether anesthesia and the second adrenal was speedily removed. In no case following the second operation did an animal receive hormonal or dietary supplement therapy.

RESULTS

Experiments on the induction of estrus with menopause urine extract. As an initial trial dosage level, 4 cats were injected with 48 R.U. of gamone per animal per day. Of these, only 3 came into heat. As shown in table 1, *cat* 2, 3 and 4 showed the first signs of estrus 7 or 8 days after the first injection was given. On the other hand, *cat* 1 never exhibited a cornified vaginal smear although she received 11 injections over a period of 16 days. In the small ovaries there were signs of follicular atresia, and the uterine histology was typical of anestrus. The lining epithelium of the uterus was extremely low, and the epithelial cells of the endometrial glands were inactive and thin, with little cytoplasm surrounding the large nuclei. Degeneration of the Graafian follicles in the ovaries was indicated by the occurrence of parthenogenetic cleavage of the ova in some instances (20).

The 3 estrous animals received single injections of follutein[3] on the second or third day of heat and all were killed after varying lengths of time (table 1) in order to look for ovulation points. The ovaries of *cat* 2, killed 51 hours after the injection of follutein, macroscopically showed no blood points or obvious corpora lutea, but there were as many as 7 large, bulging follicles. The 2 remaining estrous animals in this group (*cat* 3, 4) were allowed to live 4 and 5 days, respectively, following follutein injection. In both instances ovulation and luteinization had occurred (fig. 1). When sectioned, these ovaries showed fresh ovulation points. In addition the uteri were hypertrophied and hyperemic, and sections indicated that the animals were in early pseudopregnancy. Both endometria, into which small crypts extended, were filled in the basal region with numerous coiled glands, but the lack of complexity in the crypt formation was indicative of the earliness of the luteal phase. The lining epithelium of the enlarged glands was greatly increased in height, and the lumena of the glands were almost entirely occluded. The nuclei were basal in the tall columnar cells and the cytoplasm was clear. Similarly, the lining epithelium of the uterine lumen was hypertrophied (fig. 4).

In order to determine more accurately the dosage of gamone which would effectively produce estrus and mating reactions, it was decided to perform a series of ex-

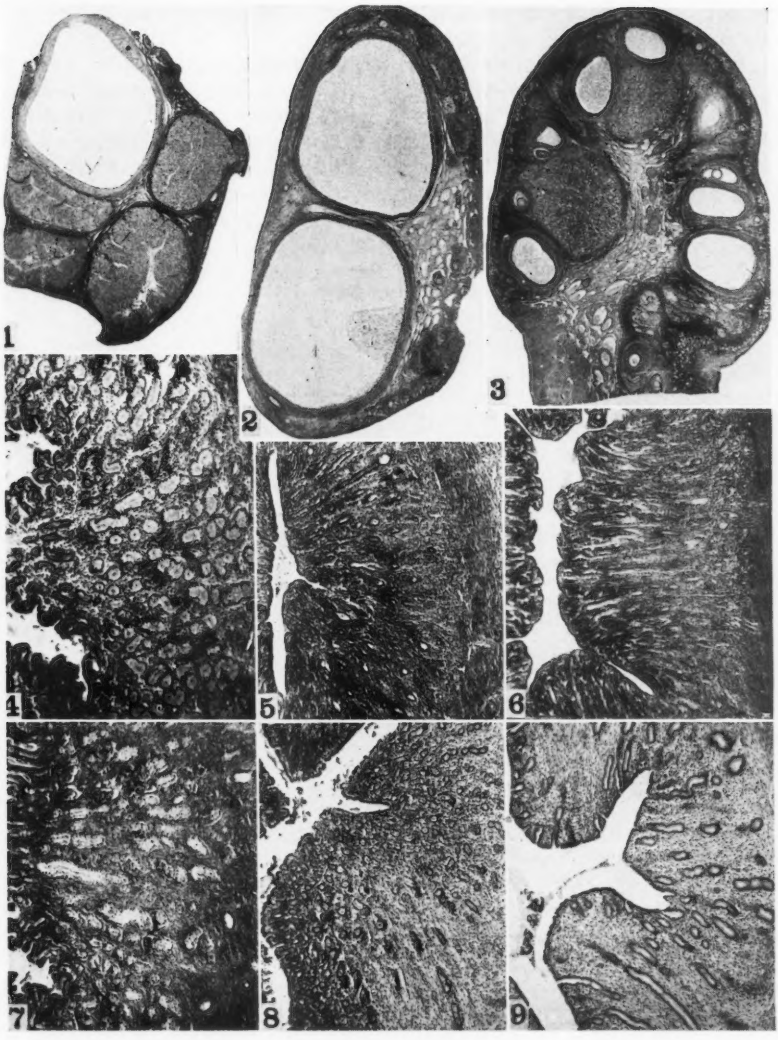

Fig. 1. Ovary of *cat 4* on the fifth day following injection of follutein after gamone induced estrus, showing luteinization of follicles and evidence of 2 ovulations. ×5. Fig. 2. Ovary of *cat 32* after 3 days of estrus induced by 4 injections of gamone. A corona radiata is present. ×9. Fig. 3. Ovary of *cat 19-C*, untreated, diestrous control, showing old, regressing corpora lutea and young, growing follicles. ×9. Fig. 4. Endometrium of *cat 3* 4 days after an injection of follutein during a gamone induced estrus. There are some signs of secretion near the uterine lumen. ×37. Fig. 5. Endometrium of *cat 21-A* 35 days after removal of the second adrenal gland, showing the regressive state and secretory exhaustion. ×37. Fig. 6. Endometrium of *cat 19-C*, untreated diestrous control in which the enlarged capillaries, very low lining epithelium, and extreme smallness of glands all indicate the inactive state of the uterus. ×37. Fig. 7. Endometrium of *cat 23-A* 22 days after removal of the second adrenal, showing a typical late pseudopregnancy. ×37. Fig. 8. Endometrium of *cat 20-A* 26 days after removal of the second adrenal, showing still a third stage in regression from pseudopregnancy. ×37. Fig. 9. Endometrium of *cat 32* with simple gland formation typical of the condition during a gamone induced estrus. ×37.

periments in which the dosage level was based on the weight of the cat. Inspection of the 4 cases described above reveals that the 3 animals which came into extrus received 18 to 20 R.U. per kg. per day. Bearing this in mind, then, *cat* 5 was given 10 R.U. per kg. per day, *cat* 6, 15 and *cat* 7, 20. As shown in table 1, the 2 animals on the lowest dosages, i.e., 10 and 15 R.U. per kg. per day, did not exhibit estrous vaginal smears or typical mating reactions although they were injected for long periods. On the other hand, *cat* 7 came into estrus after only 7 injections over an 8-day period. Since 20 R.U. per kg. per day appeared to produce effectively follicle stimulation, no further attempt was made to delimit or determine more accurately the dosage.

TABLE 1. EFFECT OF VARIOUS GAMONE DOSAGES, IN SOME CASES FOLLOWED BY FOLLUTEIN, ON MATURE FEMALES

Cat No.	Wt.	No. of inj.	Inj. period	Daily dose	Cornified smear	Estrus	Follutein inj.	Dose follutein	Remarks
	kg.		*days*	R.U.	*day*[1]	*days*	*day*[1]	R.U.	
1	3.0	11	16	48	—	—	—	—	No estrus
2	2.3	5	6	48	7	4	8	167	In estrus when killed
3	2.6	5	6	48	7	2	9	150	8 ovulation points
4	2.6	6	7	48	8	5	9	150	19 ovulation points
5	2.4	21	22	24	—	—	—	—	No estrus
6	2.9	17	18	45	—	—	—	—	No estrus
7	2.6	7	8	52	9	7	13	150	27 ovulation points
9	2.8	5	6	56	7	6	9	150	Pseudopreg. day 26[1]
18	2.4	8	8	48	9	6	—	—	In estrus at autopsy
32	1.9	4	4	40	5	3	—	—	In estrus at autopsy
36	2.8	6	6	56	7	7	—	—	Mated 3 times
37	2.8	6	6	56	7	7	—	—	Mated once
38	3.1	4	5	60	6	3	—	—	Mated 6 times
17	3.1	7	8	60	—	—	—	—	Preg., aborted day 9[1]

[1] No. of day after the first injection.

Animal 9 was injected for 6 days; she came into heat and was given 150 R.U. of follutein. The ovaries of this cat, after 26 days of pseudopregnancy, showed a high degree of luteinization, and the endometrial development was comparable to that of a pregnant animal. Whether the corpora lutea resulted from ovulated follicles or the luteinization of unovulated follicles made no difference in the production of the pseudopregnant type of uterus. Foster and Hisaw (18) found that both types of corpora lutea were physiologically active.

That ovulation could occur after mating a cat in gamone induced estrus is indicated by the presence of fresh ovulation points on the ovarian sections from *cat* 18 which was killed on the sixth day of estrus, 3 days after mating.

A third case of note in this connection is *cat* 32 (table 1). Eight days after the animal had passed through a spontaneous estrous period, gamone injections were started. Four daily injections sufficed to bring the animal back into estrus again. The short injection period might be expected since it has been reported that the cat will naturally go through a series of short diestrous cycles when isolated from the male (15). On the third day of estrus the animal was killed. The ovaries were enlarged and bulging with a total of 16 large follicles. Sections indicated that oögenesis was suspended and in at least one instance the ovum was found detached from the thin stratum granulosum (fig. 2). The uterus was only slightly enlarged and the condition of the endometrium was truly estrus-like (fig. 9).

In addition to the individual cases described above, 12 other female cats were brought into estrus with gamone and mated with a normal male. Nine of these cats were unilaterally adrenalectomized in preparation for the study of the effects of pseudopregnancy on their life-span after complete adrenalectomy. Among these cats, 4 became pregnant.

Three mid-winter cats were killed as anestrous controls, and the stages of ovarian and uterine development are pictured in figures 3 and 6, respectively.

Experiments on the survival of adrenalectomized, pseudopregnant cats. In this group of experiments 10 animals were employed. Nine of them were brought into heat artificially while the other animal (*28-A*), spontaneously came into estrus during the interval between the removal of the right and left adrenals. In order to allow ample time for the complete recovery of the animals from the effects of the first operation, from 17 to 64 days were allowed to elapse before the second operation. During this interval the animals received gamone injections and were mated (table 2).

For the most part the diet of these cats consisted of a good grade pink salmon. This, however, was supplemented with Wilsco cat and dog food and fresh milk, but these were offered only once or twice a week. During the survival period, if both salmon and Wilsco were refused, fresh kidney or scraps of roast beef were placed before the animals. As a rule food was available to the cats at all times. At no time in any of the experiments were injections of salt given, nor was any salt supplement administered in the diet, since it is well known that such treatment may significantly lengthen the life of adrenalectomized animals. Furthermore, no attempt was made to render the diet potassium-free.

The essential data on each animal throughout the experiment is shown in table 2, and a condensed protocol, showing the day-to-day treatment and condition of *cat 21-A*, is given below.

Protocol of cat 21-A. Adult female. Received Feb. 2; weight 3.1 kg. Feb. 4, right adrenal gland removed and upon laparotomy non-pregnancy was diagnosed. Feb. 9, gamone injections begin; smear made up of large and small epithelial cells, no leucocytes. Feb. 14, smear cornified after 5 injections of gamone. Feb. 19, last day of estrus. Mated only once (on Feb. 14th) but was strongly stimulated with glass rod while taking smears. Feb. 22, left adrenal gland removed under ether; weight 3.1 kg. Mar. 6, weight 3.0 kg. Mar. 11, weight 2.98 kg. Mar. 16, weight 3.0 kg. Mar. 20, bloody discharge from the vagina. Mar. 21, weight 3.0 kg. Mar. 26, showing signs of adrenal insufficiency; weight 2.8 kg.; no further vaginal discharge. Mar. 29, animal cachectic, thin, sluggish, body temperature low, not eating. Killed and autopsied. Two amorphous masses of what appeared to be degenerating blood clots in the uterus. Total survival 35 days.

In general the histology of the ovaries and uteri of the pseudopregnant animals was much the same as that described above in the section on induction of estrus and pseudopregnancy in normal cats. In some instances, however, the uterine endometrial glands appeared to have completed their secretory phase at the time of death from adrenal insufficiency (fig. 5). The ovaries were all well luteinized, but the corpora lutea in animal *21-A* definitely appeared to have reached a stage of decreased function. In those animals with shorter survival periods, the amount of uterine and ovarian regression from the pseudopregnant condition was markedly less than in the case of the 35-day cat, *21-A*. Furthermore, the amount of myometrial development was greater in the uteri of the cats surviving less than 35 days. It is possible that conditions existed in cat *21-A* which allowed it to live a few days beyond the functional life of the corpus luteum. On the other hand, it is also possible that factors other than adrenal insufficiency tended to shorten the lives of those cats which still had functional corpora lutea and hence a supply of the life maintaining luteal hormone, progesterone.

The average survival time of these 4 pregnant and 6 pseudopregnant, adrenalectomized cats was 23.2 days. This life-span is significantly longer than that recorded by various workers who have studied adrenalectomized cats. Some of the figures cited in the literature for both male and female average survivals are as follows: Rogoff and Stewart (5), 11 days in a series of 46 male animals and 10.75 days in a series of 20 female animals; Britton (21), 7.5 days; and Swingle and Pfiffner (22), 7.7 days using 38 animals and 8.6 days in a series of 100 animals. The cats in the experiments described here were operated and cared for in the same laboratory and under

TABLE 2. EFFECT OF GAMONE INJECTIONS AND MATING PREVIOUS TO COMPLETE ADRENALECTOMY IN MATURE FEMALES

Cat No.	Wt.	No. of inj.	Daily dose	Estrus	No. times mated	Interval between operations	Survival period	Remarks
	kg.		R.U.	*days*		*days*	*days*	
28-*A*	2.3	—	—	6	13	47	20	Spontaneous estrus, 5 days Pregnant, 4 embryos
8-*A*	3.2	5	64	5	8	64	20	Pregnant, single embryo
40-*A*	3.1	4	60	2	3	20	24	Pregnant, 7 embryos
41-*A*	3.2	4	64	5	7	18	22	Pregnant, single embryo
20-*A*	2.9	5	58	6	5	18	26	Pseudopregnant
21-*A*	3.1	5	61	6	1	18	35	Pseudopregnant
23-*A*	3.3	4	66	6	13	17	22	Pseudopregnant
27-*A*	2.2	5	45	5	10	28	21	Pseudopregnant
30-*A*	3.0	5	56	6	14	21	28	Pseudopregnant
39-*A*	2.9	4	60	5	5	20	14	Pseudopregnant

conditions identical with those employed by Swingle and Pfiffner for their 138 animals.

The effect of adrenalectomy upon the pregnant cat. Four attempts were made to remove the adrenals of pregnant cats. The series is small and the results are inconclusive since it was impossible to determine the exact stage of the pregnancy at the time of operation. In addition, the greater susceptibility of the pregnant animals to such manipulation renders it fairly difficult to control the experiments properly. One cat aborted 25 hours after the last operation, while another failed to eat and lived only 6 days. The remaining 2 animals survived 10 and 18 days, respectively. In general these results agree with those previously reported. Corey (3, 4) obtained a maximum survival of 10 days. Rogoff and Stewart (5) had one cat, out of a series of 7, that lived 18.75 days, but the average of the group was not above that of the controls.

DISCUSSION

A typical estrus can be induced in the female cat by intraperitoneal injection of an extract of menopause and castrate urine, gamone, as indicated by the facts that a cornified vaginal smear is produced; uterine hypertrophy occurs; the animals will mate; and, finally, such estrus is followed by a typical pseudopregnant period. The pseudopregnancy may be initiated by the animal's own pituitary, which has been stimulated by mating (23), or by the injection of an extract of pregnancy urine, follutein.

Since the life-span of the pregnant or pseudopregnant, adrenalectomized cat is dependent upon the endogenous supply of progesterone, the length of life of the corpus luteum is important. The stage of uterine development or regression at autopsy gives some insight into the luteal activity at that time. A comparison of the uterine conditions in animals 21-*A*, 20-*A* and 23-*A*, whose survivals were 35, 26 and 22 days,

respectively, shows that: (*a*) the 35-day uterus is past the secretory stage and is regressing (fig. 5); (*b*) the 26-day uterus is still fairly large but the endometrium has regressed almost to the same extent as the former (fig. 8); whereas, (*c*) the 22-day uterus is in a late secretory state with little or no regression (fig. 7). The histology of this 22-day uterus is identical with that pictured by Foster and Hisaw (18, fig. 11) for the late secretory phase of a cat 22 days in artificially induced pseudopregnancy. These data indicate that between 22 and 26 days after the beginning of the luteal phase of the ovary, the endometrial development begins to decline. However, there are indications that several days are required for a complete return to the resting state. Gros (16) reported that uterine regression after pseudopregnancy was not complete until the 33rd to 40th day after ovulation. Foster and Hisaw (18) obtained positive physiological tests indicating that pseudopregnancy induced by pituitary hormones lasted 40 to 44 days.

Since the corpus luteum of the cat ovary continues to be an active, secreting gland for 22 to 40 days after ovulation, it seems likely that it would prevent the onset of adrenal insufficiency for some time, unless the cat is especially peculiar in its physiology. Therefore, by removing the source of adrenal cortical hormone from the animal at the very beginning of the active phase of the corpus luteum, it has been possible to utilize the total progesterone secretion. When this is done, there is apparently no difference in the survival periods of pregnant and pseudopregnant animals, both of them being significantly extended. In only one instance was the life-span less than 20 days (*cat 39-A*), and the average for the group (23.2 days) was almost 3 times as long as the figures given for the survival of adrenalectomized males and non-pregnant females. The conclusion is thus reached that the pregnant or pseudopregnant cat does not differ essentially from the dog in its reaction to adrenalectomy. The effect of the long luteal phase upon the adrenalectomized dog is merely easier to demonstrate because of its great length, as compared with that of the cat.

SUMMARY

An extract of menopause and castrate urine, gamone, has been found effective in inducing estrus in mature female cats. A dosage level of 20 R.U. per kg. body weight per day invariably produced a cornified vaginal smear and mating reactions. Moreover, a single injection of 150 R.U. of follutein (pregnancy urine hormone), during gamone induced estrus, produced ovulation and pseudopregnancy. Out of a total of 12 mated animals, 4 became pregnant. These data support previous evidence that gamone is a follicle stimulating substance.

In a series of 10 unilaterally adrenalectomized female cats, estrus was induced in 9 cases, whereas the remaining animal came into spontaneous estrus. All animals were mated before removal of the second adrenal. Subsequently it was ascertained that 4 of the cats so treated were pregnant and 6 were pseudopregnant. Only 1 animal in 10 survived bilateral adrenalectomy less than 20 days, whereas the maximum life-span obtained was 35 days. The average for the series was 23.2 days, which is almost 3 times as long as the untreated adrenalectomized cat usually lives. Thus, it appears that the secretion of progesterone from the corpora lutea of the pregnant and pseudopregnant animals exerts an ameliorating effect upon the symptoms of adrenal insufficiency. In this respect the cat and the dog apparently do not differ.

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SKELETAL CHANGES IN RATS RECEIVING ESTRADIOL BENZOATE AS INDICATED BY HISTOLOGICAL STUDIES AND DETERMINATIONS OF BONE ASH, SERUM CALCIUM AND PHOSPHATASE[1]

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SEVERAL investigations of estrogens in relation to skeletal growth and composition have shown that repeated injections of the hormones over long periods of time appear to cause a condition of generalized hyper-ossification (1–9), except in the pubic symphysis (2). The principal observations have been made on mice (2, 4, 7, 8), chickens (6, 9) and guinea pigs (5). The only known studies made on rats are those of Zondek (1), Macola (10) and Bach (11). The present investigation was made to secure (*a*) definite information concerning the skeletal response of rats to estrogen treatment and (*b*) to examine some of the factors which might affect the skeletal response of rats to excesses of estrogens, thus providing some basis for judgment on the nature of the relationship of estrogen to the skeletal tissues.

PROCEDURE

Rats from this colony were used. In each experiment they were paired as carefully as possible as respects litters, sex and weight. They were housed in clean metal cages. Rats used in the paired feeding experiments were kept in individual cages, but in the other studies there were three or four animals per cage. Approximately 200 rats were used ranging in age from about 30 to 650 days. Two different diets were used. Their composition is given in table 1.

TABLE 1. COMPOSITION OF DIETS USED.

Stock colony (5)		No. 23	
Wheat	25.0	Casein, acid washed	20.0
Yellow maize	25.0	Dextrin	30.0
Oats	28.5	Sucrose	25.0
Flaxseed oil meal	10.0	Vegetable fat (Crisco)	10.0
Casein, crude	10.0	Yeast	8.0
$CaCO_3$	0.5	Salts #51[1]	5.0
NaCl	1.0	Cod-liver oil	2.0

[1] McCollum salt mixture: $CaCO_3$ 24.58, KCl 16.40, NaCl 8.20, $NaHCO_3$ 11.48, MgO 3.28, ferric citrate 8.20, and KH_2PO_4 27.86.

The purified diet, No. 23, was employed in the paired feeding studies to permit more satisfactory regulation of the food intake.

The estradiol benzoate[2] was administered 2 times weekly by subcutaneous in-

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jection in sesame oil. The control animals were given similar injections of oil only.

The left femur of each rat was freed from the bulk of soft tissue and boiled for 3 minutes in distilled water. This facilitated uniform removal of all soft tissue. The bones were extracted 24 hours with ethyl alcohol and then 24 hours with ethyl ether, using a Soxhlet extractor. Following this they were dried for 24 hours at 100° C. and ashed 16 hours at 550° C. The serum calcium and magnesium were determined by the Larson and Greenberg (12) and Greenberg and Mackey (13) methods respectively. The blood was obtained by cardiac puncture, or decapitation, following ether anesthesia. The King and Armstrong (14) method for alkaline phosphatase, as modified by Weise et al. (15), was used to determine serum and bone phosphatase. The left tibia and fibula were used for the latter. The fresh bones from each rat, after being freed from soft tissue, were lightly rinsed with distilled water, ground in a mortar, and transferred to a small flask, using distilled water. The weight of each sample was adjusted to 25 gm. Chloroform was used as a preservative.

In the histological studies bones were taken from a total of 64 rats which were about equally divided as to sex. The animals may be roughly divided into 3 groups, depending on their age at the time treatment was begun: young (4 to 5 weeks), adult (10 to 25 weeks), and senile (over 1 year).

The proximal end of the tibia was fixed in formalin, decalcified in nitric acid, embedded in paraffin, and stained with hematoxylin and eosin. In some of the animals the distal end of the femur was embedded in celloidin and stained with hematoxylin and eosin after fixation and decalcification. Sections of the gonads and accessory sexual organs were also examined microscopically to be certain that the estrogen was having the desired effect.

RESULTS

Tables 2 to 6 summarize the bone ash and chemical data. In order to conserve space only mean values are presented, with values for t (16), the expression of statistical significance of a mean difference.

Table 2 shows that groups of young rats (30 to 37 days old) treated with the estrogen for periods of 17, 28, 44 and 104 days respectively, attained a definite skeletal response after 44 days as shown by an increase in the concentration of bone ash ($t = 4.12$). With the number of animals used in these groups t must be greater than about 2.3 in order to be quite certain that the differences are truly significant. It is indicated that even by the end of 17 days the concentration of bone ash in the estrogen treated rats tends to be higher than in the controls. After 104 days the difference does not appear to be any greater than at the 44-day level. As compared with their controls, the total amount of ash was less in the rats receiving estrogen for 104 days (t 3.06). This effect was indicated in the other age groups, but it lacked statistical significance.

In these chemical data there are no clear indications of any sexual difference in the rate of development of bone changes but there is a suggestion that females treated 44 days have a greater increase in the concentration of bone ash than males similarly treated. The well-known normal sexual difference in the concentration of bone ash is clearly indicated in tables 2 and 3.

Table 3 presents a summary of experiments designed to determine the effect of estradiol benzoate on the calcification of bones of rats of different ages. In the literature no significance appears to have been attached to the possible relationship between age at the beginning of estrogen treatment and the occurrence of modifications in the skeletal structures. Therefore, the data in table 3 should be of special interest. As indicated in table 3, young rats showed very definite increases in the concentration of bone

ash, following estrogen treatment, for 44 to 106 days, but rats 133 to 650 days old the beginning of treatment showed no change after receiving estradiol benzoate for 37 days, in one case (650-day-old rats), and 125 days in the other (133- and 217-day-old rats). The hormone was not without effect on the bodies of the older animals because the treated rats showed essentially as much depression of growth, or body weight, as the younger treated animals. Also, the gonadal changes in the older rats showed that the hormone had a decided systemic effect.

TABLE 2. EFFECT OF ESTRADIOL BENZOATE ON CALCIFICATION OF BONES OF 30- TO 37-DAY-OLD RATS TREATED DIFFERENT LENGTHS OF TIME
(Diet No. 23)

No. of rats and sex	Treatment	Days treated	Weight increase	Mg. ash (femur)			% ash (femur)		
				Mean	Av. deviation from mean	t	Mean	Av. deviation from mean	t
			gm.						
4♀	2000 U. E.	17	39	105.7	10.9		58.2	1.6	
4♀	oil	17	51	103.0	7.3	0.31	57.2	1.6	0.69
4♂	2000 U. E.	17	48	111.7	3.5		56.9	0.5	
4♂	oil	17	64	106.7	7.6	0.86	56.5	0.7	0.62
Total (8)	2000 U. E.	17	44	108.7	7.2		57.6	1.1	
Total (8)	oil	17	58	104.9	7.5	0.77	56.9	1.2	1.26
4♀	3200 U. E.	28	50	146.8	19.2		61.7	1.4	
4♀	oil	28	83	157.8	5.2	0.79	61.3	1.7	0.29
4♂	3200 U. E.	28	90	173.7	21.2		60.6	1.1	
4♂	oil	28	134	167.3	13.4	0.38	59.5	1.8	0.81
Total (8)	3200 U. E.	28	70	160.3	20.2		61.2	1.2	
Total (8)	oil	28	109	162.6	9.3	0.19	60.4	1.7	0.81
5♀	5200 U. E.	44	84	186.6	4.4		65.8	0.7	
5♀	oil	44	125	196.9	17.4	0.87	63.1	0.7	4.69
4♂	5200 U. E.	44	112	224.0	16.6		64.4	1.1	
4♂	oil	44	196	226.8	32.7	0.12	62.4	0.7	2.16
Total (9)	5200 U. E.	44	98	205.3	9.8		65.1	0.9	
Total (9)	oil	44	161	211.9	24.2	0.48	62.8	0.7	4.12
7♂	12000 U. E.	104	121	322.1	25.2		68.5	0.8	
7♂	oil	104	260	367.6	18.6	3.06	66.5	0.6	3.78

The growth inhibition in young animals treated with estrogens has been noted by various workers (1, 17). Whether this, with the accompanying decrease in food consumption, has any effect on the degree of skeletal changes has not been recorded in the literature. Table 4 presents the results of experiments intended to answer this question. In these studies each estrogen treated rat had 2 controls, one being restricted to an amount of food equal to that eaten by the treated animal, and the other being allowed to eat ad libitum. After about 2 weeks of estrogen treatment the food consumption of rats began to decline. During the course of a few weeks the difference amounted to 10 to 20%. Consequently, the "food restricted" controls received considerably less food than the ad libitum controls. Nevertheless, the former controls gained in weight considerably better than the estrogen injected animals, indicating a more efficient utilization of the food. The gain in weight was intermediate between that of the treated rats and the controls allowed to eat at will. However, the concentration of bone ash tended to be somewhat less in the food restricted rats than in the controls. Likewise, the total amount of ash tended to be less in the food-restricted animals. Hence, the decreased food consumption of estrogen injected rats,

TABLE 3. EFFECT OF ESTRADIOL BENZOATE ON CALCIFICATION OF BONES OF RATS OF DIFFERENT AGES

No. of rats and sex	Treatment	Diet	Age when started	Days treated	Weight increase	(Mg. ash femur)			% ash (femur)		
						Mean	Av. deviation from mean	t	Mean	Av. deviation from mean	t
			days		*gm.*						
5♀	5200 U. E.	23	30	44	84	186.6	4.2		65.8	0.7	
5♀	oil	23	30	44	125	196.9	17.4	0.89	63.1	0.7	4.63
6♀	4400 U. E.	S	650	37	−18	310.1	15.7		67.6	1.0	
6♀	oil	S	650	37	1	291.3	12.5	1.69	67.3	0.6	0.50
7♂	12000 U. E.	23	36	104	121	322.1	25.5		68.5	0.8	
7♂	oil	23	36	104	260	367.6	17.2	3.05	66.5	0.6	3.84
5♂	12400 U. E.	23	82	106	40	388.5	41.1		68.2	0.5	
5♂	oil	23	82	106	126	383.3	24.3	0.21	66.2	0.6	5.17
3♂	10800 U. E.	23	217	125	−6	387.0	29.8		67.3	1.0	
3♂	oil	23	217	125	29	381.2	31.4	0.17	67.0	1.0	0.28
3♀	10800 U. E.	23	133	125	11	275.5	15.6		66.8	0.5	
3♀	oil	23	133	125	35	289.4	12.6	0.92	67.3	1.1	0.20
Total (6)	10800 U. E.	23	175	125	3	331.3	22.7		67.1	0.8	
Total (6)	oil	23	175	125	32	335.3	22.0	0.11	67.2	1.1	0.02

instead of accentuating the difference between concentration of bone ash in such animals compared with controls fed ad libitum, may tend to reduce the difference.

In order to form some basis for considering the nature of the changes produced

TABLE 4. EFFECT OF ESTRADIOL BENZOATE ON CALCIFICATION OF BONES OF RATS COMPARED WITH CONTROLS SUBJECTED TO (*a*) RESTRICTED FOOD INTAKE AND (*b*) FED AD LIBITUM
(Diet No. 23)

No. of rats and sex	Treatment	Age when started	Days treated	Weight increase	Mg. ash (femur)		% ash (femur)	
					Mean	Av. deviation from mean	Mean	Av. deviation from mean
		days		*gm.*				
2♀	3200 U. E.	28	26	39	127.6	0.6	62.5	0.1
2♀	oil, food restr.	28	26	62	123.7	13.3	60.2	0.4
2♀	oil, food ad. lib.	28	26	76	154.6	2.3	63.0	0.7
2♂	3200 U. E.	28	26	78	158.4	5.6	61.6	0.4
2♂	oil, food restr.	28	26	94	138.9	4.4	58.8	0.0
2♂	oil, food ad. lib.	28	26	126	159.7	1.4	61.3	1.3
3♀	5200 U. E.	28	42	85	185.7	6.8	66.2	0.5
3♀	oil, food restr.	28	42	107	165.8	18.0	63.3	1.0
3♀	oil, food ad. lib.	28	42	128	184.4	20.3	62.9	0.3
2♂	5200 U. E.	28	43	115	207.5	5.2	63.3	0.9
2♂	oil, food restr.	28	43	133	184.6	5.0	61.8	0.1
2♂	oil, food ad. lib.	28	43	176	194.1	16.6	62.3	0.2
3♀	oil, food restr.	50	122	64	246.1	14.8	68.0	0.4
3♀	oil, food ad. lib.	50	122	95	254.3	6.4	67.7	0.3
3♂	oil, food restr.	50	122	118	314.6	17.1	66.3	0.3
3♂	oil, food ad. lib.	50	122	201	376.6	5.2	67.4	1.1

in growing bone certain of the rats, used in securing the data given in tables 2 and 3, were employed to determine the alkaline phosphatase activity of blood serum and bone (tibia and fibula). Although various factors, other than those related to processes of ossification and bone metabolism, affect the level of phosphatase activity of bone and serum (18), it was believed that data on this variable might be of some significance.

TABLE 5. EFFECT OF ESTRADIOL BENZOATE ON ALKALINE PHOSPHATASE OF BLOOD SERUM AND BONE (TIBIA AND FIBULA) OF RATS TREATED DIFFERENT LENGTHS OF TIME.

No. of rats and sex	Treatment	Diet	Age when started	Days treated	Serum phosphatase (u./100 cc.)			Bone phosphatase (u./100 mg. dry bone)		
					Mean	Av. deviation from mean	t	Mean	Av. deviation from mean	t
			days							
2♂, 2♀	2000 u. E.	23	30	17	95.6	7.3		9.18	0.91	
2♂, 2♀	oil	23	30	17	97.5	7.2	0.28	7.66	0.49	2.42
2♂, 2♀	3600 u. E.	23	30	30	67.8	6.3		6.16	0.26	
2♂, 2♀	oil	23	30	30	80.6	4.0	2.43	6.37	0.22	0.94
2♂, 2♀	5200 u. E.	23	30	46	42.0	2.8		5.16	0.42	
2♂, 2♀	oil	23	30	46	57.0	2.7	5.67	4.79	0.18	1.21
7♂	12,000 u. E.	S	36	104	55.4	11.9		1.87	0.41	
7♂	oil	S	36	103	54.7	3.9	0.12	2.51	0.23	1.73
5♂	12,400 u. E.	S	82	106	52.1	8.6		1.72	0.24	
5♂	oil	S	82	106	56.7	7.9	0.69	2.05	0.24	1.79

Thirty-day-old rats treated for 17 days showed no change in the level of serum phosphatase, but the bone phosphatase activity probably was increased (table 5). The value of t in the comparison of bone phosphatase is 2.42. This means that there is only about 1 chance in 20 that the difference is not real. Thirty-day-old rats treated for 30 days showed definitely less serum phosphatase than their controls but the differences in bone phosphatase values were not significant. This same effect was found in the serum and bone phosphatase levels of rats treated 46 days. But rats that were given the stock colony diet and treated slightly over 100 days showed no effect in either the serum or bone phosphatase values. It will be noted that the amount of phosphatase (activity) decreases in both serum and bone as the rat becomes older. This is a well-known fact (19). The data appear to indicate that estrogen in excess superimposes an inhibiting effect on the normal age decrease in serum phosphatase. By the time the animal is well past 100 days of age normal growth has about ceased and the excess of estrogen is essentially without further effect on serum phosphatase.

Serum calcium was determined in a total of 56 rats. The data are given in table 6. Animals 5 weeks old when injections were begun, and treated for 23 days, showed a definite increase as compared with control rats ($t = 2.44$). Animals treated for 84 days showed a similar increase. But there was no effect in mature rats given estrogen for 125 days and in senile animals treated for 37 days.

Serum magnesium determinations were made simultaneously on all the rats except those in the senile group. In the young animals no certain difference was found although the mean values were higher in the injected rats than in the controls (table 6) There was no indication of difference in the adult rats.

MICROSCOPIC FINDINGS

Young group. This consisted of 40 animals, both males and females, which were 4 to 5 weeks old when treatment was begun. Histological studies were made at intervals of 17, 26, 44, 84 and 115 days after estrogen administration had been started. Since animals injected with large amounts of estradiol benzoate fail to gain as well as their normal controls (1, 17) the diet of some was restricted to the level of that of the estrogen treated animals.

After 17 days there was a definite histological difference between the estrogen treated animals and their controls. Gardner and Pfeiffer (3) found that testosterone

TABLE 6. EFFECT OF ESTRADIOL BENZOATE ON THE LEVEL OF SERUM CALCIUM AND MAGNESIUM OF RATS.

No. of rats and sex	Treatment	Diet	Age when started	Days treated	% ash in femur	Ca (mg./100 cc.)			Mg (mg./100 cc.)		
						Mean	Av. deviation from mean	t	Mean	Av. deviation from mean	t
			days								
7 ♀, 6 ♂	3600 U. E.	23	36	30	62.1	11.93	0.47		2.54	0.31	
7 ♀, 6 ♂	oil	23	36	30	61.1	11.22	0.60	2.44	2.16	0.15	1.83
5 ♂	7200 U. E.	23	36	84	66.3	11.58	0.56		3.04	0.09	
5 ♂	oil	23	36	84	63.1	10.59	0.53	2.52	2.68	0.37	1.88
4 ♂	10,800 U. E.	23	220	125	67.4	10.83	0.20		2.53	0.25	
4 ♂	oil	23	220	125	67.0	10.72	0.15	0.21	2.75	0.55	0.55
6 ♀	4000 U. E.	S	650	37	67.6	11.03	0.47				
6 ♀	oil	S	650	37	67.3	11.17	0.33	0.15			

propionate inhibits hyperossification due to estrogen excess, thus indicating a probable reason for the sexual difference. The changes from normal were more marked in the females than in the males. The epiphyseal cartilage plates had the same width. The columnar cartilage cells, as well as the hypertrophic cells, were alike in the two groups. There was no increase in calcified cartilaginous matrix. No increase in osteoblasts at the cartilage shaft junction could be detected in either group, nor were the bony trabeculae increased in number or thickness at the cartilage shaft junction. However, the trabeculae which extended further down the shaft were more numerous as well as thicker in the estrogen treated rats than their controls. The bone corpuscles were not increased in number. No differences in periosteal bone formation were detected. The osteoid was not excessive. The marrow in each group showed no change. Thus there was apparently an increased amount of bone just beneath the cartilage shaft junction, as well as a decreased destruction of trabeculae, since in the controls the number of trabeculae diminished in normal fashion from epiphysis to shaft while in the estrogen treated rats these trabeculae persisted.

At 26 days the changes were even more marked (fig. 1, 2). Again the females showed greater degrees of change than the males. At this period the factor of partial inanition had to be considered as well, so comparison was made with the food restricted controls. The cartilage plates of the estrogen treated animals were a little narrower than their controls on the restricted food intake while the latter were narrower in turn than those of the animals fed ad libitum. There was perhaps a slight decrease in the number of columnar cartilage cells as well as hypertrophic cells in the estrogen treated animals when compared with their restricted controls. As in the animals injected for 17 days, there was a dense deposition of bony trabeculae beneath

the cartilage shaft junction and these trabeculae extended down the shaft to a distance which correlated with the beginning of estrogen treatment. In contrast, the trabeculae in both groups of controls were apparently undergoing the usual normal destruction.

At 44, 84 and 115 days the end of the shaft was even more dense (fig. 3, 4, 5, 8). The epiphyseal cartilage was still growing though not so rapidly as the normal controls. The restricted animals likewise showed a slowing of growth of the cartilage. No differences in periosteal bone formation were found. The change was taking place at the growing end of the bone and this dense bone persisted down into the shaft apparently because destruction was retarded to some extent, at least.

Adult group. This consisted of 18 animals, both males and females, whose ages ranged from 10 to 20 weeks when estrogen treatment was begun. The injections were continued for 18 weeks.

Microscopially, there was a definite difference between the estrogen treated rats and their controls. However, it was not as marked as in the younger group and this was undoubtedly associated with the slowing up of bone growth which occurred as age progressed. The cartilage plates were narrower than in the foregoing group and the proliferative cells were fewer. In the controls there were only a few stout trabeculae of bone while in the treated animals these trabeculae were more numerous. In both groups most of the shaft, except near the epiphysis, was completely free from trabeculae. Again, no excessive periosteal bone formation was in evidence. The cellular marrow of the two groups was identical.

Senile group. This consisted of 6 animals that were almost 2 years old. Three were treated with estrogen for 37 days (fig. 6, 7). No changes could be detected, probably because epiphyseal growth had practically ceased. Owing to this, scarcely any change could be expected in the relatively short period of treatment. No periosteal new bone was noted in the experimental animals, nor was there any in the shaft save at the cartilage shaft junction where there appeared to be a slight excess of bone over that in the controls.

DISCUSSION

The chemical, histological and x-ray evidence reported here indicates that the skeleton of growing rats subjected to a continued excess of estradiol benzoate is affected primarily by a decreased physiological destruction of bony trabeculae. However, the number and thickness of the individual trabeculae beneath the cartilage shaft junction are greater in the estrogen treated animals. The possibility that there is some increase in osteoblastic activity must not be entirely disregarded. However, it is difficult to understand how this could occur because the total dry weight and the ash weight of the bone is reduced. Since the alterations in bone occur principally in the epiphyseal portions it is not surprising that the changes in concentration of ash should be less striking than the microscopic changes. Probably determinations of ash concentration in the epiphyses would have revealed considerably larger changes than in the whole bones.

Landauer, Pfeiffer, Gardner and Man (6) produced marked hyperossification and calcemia in male chickens 6 to 7 months old at the time treatment was begun. Since that age in cocks probably is comparable to a considerably younger physiological age in rats the observation is not necessarily in disagreement with our failure to find very appreciable skeletal changes in mature or senile rats. Moreover, calcium metabolism is so different in birds, as compared with mammals, that chickens and rats should not be expected to respond in a similar manner to excesses of estrogen.

There is no doubt that the older rats were receiving enough hormone to con-

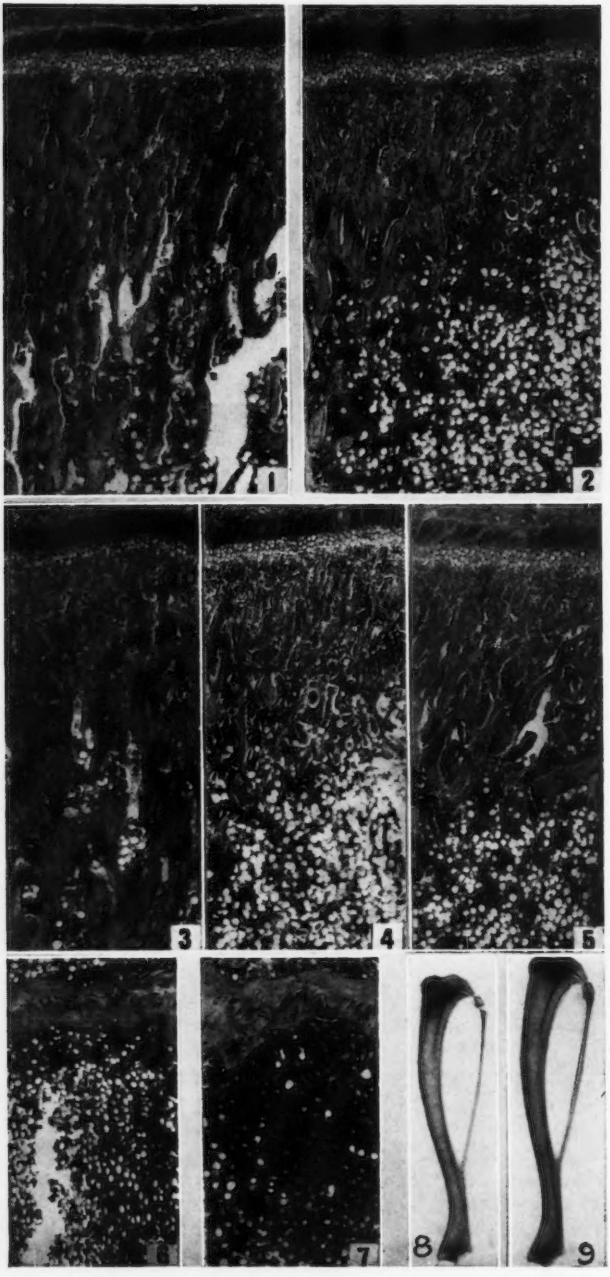

Fig. 1. Bone from young female rat which had been treated with estrogen 26 days. Fig. 2. Bone from control female rat of same age as figure 1 whose diet had been restricted. Fig. 3. Bone

stitute an excess. Reproductive organs and body weight were affected in typical fashion. Jaffe, Bodansky and Blair (20) noted that young guinea pigs were more susceptible than adults to the effect of single or repeated doses of parathormone, as shown by the bone changes. Regardless of dosage level, the evidence indicates that very appreciable skeletal effects should not be expected in adult rats treated with estrogen since the responsiveness of the skeleton to estradiol benzoate appears to be dependent upon an active anabolic state in the bone.

The increase in concentration of bone ash found by Gardner et al. (2, 7) in mice is definitely greater than we have produced in any of the rats studied. Whether this is attributable to species difference, or to differences in experimental procedure cannot be decided with the evidence available. The mice at Yale were subjected to considerably longer periods of treatment with estrogen than most of the rats used in this study. Possibly the mice received relatively larger doses of estrogen. They were given 100 to 1000 I.U. of estradiol benzoate per mouse weekly (2). Our animals received 800 I.U. per rat weekly. Since adult and senile rats apparently are not as responsive to estrogen as young rats it would seem that much of the skeletal alterations must occur before maturity is reached, even though treatment is prolonged.

As Morris and Peden (18) have pointed out, the significance of serum phosphatase has been scarcely determined. It is generally regarded that the factor which is finally responsible for the level of serum phosphatase in disturbances of bone metabolism is the relationship between cellular activity of the osteoblasts, the effective supply of calcium and phosphorus, and the ability of the cells to utilize these elements. But some important evidence indicates that serum phosphatase is as much affected by the status of carbohydrate metabolism as bone metabolism (21). Therefore, the unspecificity of serum phosphatase, as a thoroughly reliable indicator of bone metabolism, should be recognized. However, the concentration of the enzyme, or its activity, in bone itself is generally regarded as somewhat indicative of the osteoblastic activity (19). Thus the data reported here on the phosphatase activity of both serum and bone, together with the microscopic findings, may be of value in interpreting the general effect of estrogen on bone.

Our findings are not in agreement with those of Folley (22) who observed that serum phosphatase of a few lactating cows treated with estrogen rises and that calcium falls. Our microscopic and chemical evidence suggests that the degree of osteoblastic activity of bone is scarcely affected by an excess of estrogen, except possibly in the first 3 weeks when the activity may be slightly increased. In relation to the depression in the serum phosphatase level, after a few weeks of estrogen treatment, it is interesting that Barnes and Munks (23) found an increase in the serum phosphatase level of infants during the first few weeks of life. It is reported that estrogenic substance occurs in the urine of infants at birth, but it disappears shortly thereafter (24).

The data on serum calcium and magnesium are of some interest in view of the unsettled question: does estrogen in excess affect the concentration of serum calcium? Our data show that rats in which definite skeletal changes were produced also showed an increase in the concentration of serum calcium. The validity of this effect is further indicated by the tendency of serum magnesium to be increased. Levin and Smith (25), in studies of rats, rabbits and monkeys concluded that their results failed to

FROM YOUNG FEMALE RAT WHICH HAD BEEN TREATED WITH ESTROGEN FOR 44 DAYS. Fig. 4. BONE FROM CONTROL FEMALE RAT OF SAME AGE AS FIGURE 3 WHOSE DIET HAD BEEN RESTRICTED. Fig. 5. BONE FROM CONTROL FEMALE RAT OF SAME AGE AS FIGURES 3 AND 4, FED AD LIBITUM. Fig. 6. BONE FROM SENILE FEMALE RAT WHICH HAD RECEIVED ESTROGEN TREATMENT 5 WEEKS. Fig. 7. BONE FROM CONTROL FEMALE RAT OF SAME AGE AS FIGURE 6. Fig. 8. X-RAY OF BONE FROM ESTROGEN INJECTED (44 DAYS) RAT. Fig. 9. X-RAY OF CONTROL RAT OF SAME AGE AS FIGURE 8. (Photographs by Mr. Milton Kougl.)

confirm Riddle and Dotti's (26) claim that estrogens raise the level of serum calcium. In all instances their animals treated with estrogens had slightly more serum calcium than the controls. That finding is similar to ours. We regard the difference as significant because statistical analysis of the data makes that conclusion unequivocal.

The histological observations would seem to be in agreement with the x-ray studies of Zondek (1) on rats. In the literature the histological descriptions of changes in the bones of various species (pigeon, mouse, guinea pig) produced by estrogens, are not entirely in agreement with one another or with the observations we have made on rats. Thus, Pfeiffer and Gardner (4), studying pigeons, have described the production of newly formed endosteal medullary bone in the shaft which eventually practically filled the marrow cavity. In birds the calcium metabolism is so different from that in mammals that extensive production of new bone in the diaphysis is not surprising. Pfeiffer and Gardner (2), in studies on mice treated with estrogen, described an extensive endosteal bone production in the diaphysis with practical obliteration of the marrow space.

Sutro (8), using immature mice treated with estrogen, found an increase in the density of the bone at the zone of provisional calcification and as growth progressed this advanced into the diaphysis. No periosteal new bone formation was noted. Silberberg and Silberberg (5), using immature guinea pigs, have studied the effects of estradiol benzoate on bone and cartilage. They observed "a temporary increase in the trabeculae of the epiphyses and diaphyses during the first four weeks." They did not observe "any appreciable apposition of bone either periosteally or endosteally sufficient to cause thickening of the compact bone or narrowing and occlusion of the marrow cavity." They referred to a "premature aging" of the cartilage. It appears probable that some of the apparent differences in the recorded effects of estrogens on the skeleton are due to differences in species, age of animals when hormone administration was begun, and length of treatment.

SUMMARY

More than 200 rats, from 30 to 650 days of age, have been given subcutaneous injections of estradiol benzoate over intervals of time varying from 17 to 125 days. The effects on the skeleton have been studied by means of histological, x-ray, and chemical technics. The latter included determinations of total bone ash, percentage bone ash, bone phosphatase, serum phosphatase, and serum calcium and magnesium. The paired feeding technic was used in certain experiments.

Histological changes, consisting of an increase in density at the growing end of the bones, become detectable in young rats as early as 17 days after the beginning of treatment with estrogen.

The evidence indicates that estrogen causes a decrease in normal destruction of bony trabeculae just beneath the cartilage shaft junction. There is also some increase in osteoblastic activity at the cartilage shaft junction, but not elsewhere. This increases the density of the epiphyses and apparently accounts for the increase in concentration of ash in the whole bone. However, the total amount of bone is reduced owing to decreased growth in length.

The skeletal changes are greater in females than in males.

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ADMINISTRATION OF RAT THYMUS TO PREGNANT RATS AND THE LACK OF EFFECT IN SUCCESSIVE GENERATIONS[1]

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IN 1934, Rowntree and his associates reported that daily treatment of rats with an extract of calves' thymus caused an acceleration of growth and development in the offspring of these animals. With continued treatment, the effect was found to be enhanced in each succeeding generation (1). This report met with considerable scepticism since it apparently contradicted the theory firmly established by Weismann, that acquired characters cannot be inherited. Many criticisms were directed against the work and several attempts to reproduce the phenomenon in other laboratories failed (2, 3). Nevertheless, the original group of investigators have fortified their claims by other experiments in which the same type of effect, though lesser in degree, has been obtained by weekly implantation of whole rat thymuses into rats (4, 5). Assuming the validity of these experiments, some reasonable explanation of the results should present itself. It is not necessary to assume an endocrine rôle for the thymus since it has not been shown that the effective factor is found only in this organ. It has also not been shown that the Weismannian doctrine is invalidated by these experiments since cessation of treatment for one generation resulted in an immediate return to normal growth and development in the succeeding generation.

Two facts have presumably been demonstrated by these experiments. First, some factor in the thymus extract and tissue produced accelerated growth and development and second, as long as treatment was continued, the effect in each succeeding generation was more marked.

Since, in the original experiments, treatment was continued throughout pregnancy, it seemed possible that the effect may have been registered directly on the developing fetus, perhaps by speeding up the metabolic processes involved in development. After birth the effective principle may have been transferred through the milk of the mother who continued receiving treatment. At weaning, the animal itself received treatment so that the altered metabolic state may have been retained. When this animal became pregnant, its developing fetuses then would be subject to a double effect, the environment provided by the mother plus the continued treatment given the mother during pregnancy and lactation. In this way the accruing effects in succeeding generations might be explained.

If this hypothetical explanation is sound, only the females would need treatment in order to produce the effect. Rowntree and his associates have recently indicated that treatment of the male is not necessary (6), although no data has appeared to substantiate this assertion.

The question arose as to whether or not the accelerative effect on growth and development could be produced by treatment of the females alone and only during

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the period of pregnancy. The experiment reported here was undertaken to test this point. In order to eliminate the use of an extract, the method of implantation was selected and, in order to magnify the effectiveness of this method, daily administration of ground thymus was used instead of the weekly implantation of whole thymuses, the method used by the Rowntree group.

PROCEDURE AND RESULTS

Parent generation (F_0). Six females with sperm in the vaginal smear were selected from our stock colony. This colony is not a pure strain, but has been inbred for 4 years. Each of these females was treated 6 times weekly with a saline suspension of ground rat thymus, each receiving the equivalent of one thymus per treatment. Donors were males and females, 32 to 40 gm. in weight and approximately 25 days old. The thymus tissue was ground in a small mortar with 0.9% saline until only a white connective tissue residue remained. All the rest of the suspended material was taken up into a syringe through a 21-gauge needle with sufficient saline solution added to make a total of 1 cc. Injections were made subcutaneously into the back of the animal, the site of injection being changed on subsequent days. Many of the animals showed some local reaction to the injections. Indurated areas which later ulcerated were often found. None of the original six animals showed any signs of general toxicity, however, and all delivered healthy litters on the 22nd day of gestation, which is the normal term for the animals of our colony. Treatment was discontinued on the day of delivery. This procedure was followed throughout the experiment.

F_1 generation. From the 6 original mothers a total of 38 offspring were obtained. However, in order to standardize conditions as far as possible, all experimental and control litters were reduced to 6. When possible, 3 males and 3 females were kept in the litter with the natural mother. Litters smaller than 6 were supplemented by rats of the same age from stock. The added animals were not included in the data. All litters were weaned on the 21st day. Daily weight records were kept on all animals up to the age of 50 days. Eruption of the teeth, opening of the eyes and vaginal opening were selected as criteria of developmental rate since these are sharply defined processes which are not subject to variations in the judgment of the observer.

The F_1 animals, when compared to 6 non-selected litters (of 6 each) from general stock, seemed to show some slight indications of accelerated growth, in that the average weight curve of the F_1 thymus animals was slightly higher than that of the control animals. When the weights were plotted according to daily range, the thymus animals showed a decided advantage, although there was some overlapping. No marked changes from the normal occurred in the development of the F_1 animals.

When the F_1 animals reached maturity, 5 of the surviving females were mated with litter brothers and the remaining 10 were mated with a single stock male. This latter procedure was employed in order to diminish the possibility of selective breeding. Daily vaginal smears were examined after mating and, as soon as sperm were found in a smear, the female was isolated and treated.

In the treatment of the F_1 females, another control procedure was introduced. Some of the females were treated with spleen tissue which was taken from the same donors as the thymus tissue. The amount of spleen used was equivalent to the bulk of the thymus and was prepared for injection in the same way as the thymus. In addition, some of the F_1 females were not treated. Ideally, each F_1 litter should have provided a thymus treated female, a spleen treated female and a non-treated control. Although this objective was impossible, there were 6 thymus treated females, 2 spleen treated females and 3 non-treated females of the F_1 generation which delivered

viable litters. Treatment was administered, as in the parent generation, 6 times weekly throughout pregnancy and was discontinued at term.

One thymus treated and one spleen treated female died while under treatment. One thymus treated and two non-treated females either resorbed their fetuses or gave birth to non-viable young.

F_2 generation. This generation consisted of 70 animals of which 38 were the offspring of the thymus treated mothers, 12 were from spleen treated mothers and 20 from non-treated mothers. The growth curves for these 3 groups showed variabilities. For the first 5 days the thymus group averaged slightly higher than the other 2; from the 5th day to the 50th the spleen treated animals averaged higher than the other 2; up to the 35th day the non-treated group averaged the lowest, but from the 35th day to the 50th the thymus animals lagged considerably behind the others in weight gain. Compared with the weight curve for the stock controls used in the first generation plus an additional 6 litters run at the same time as the F_2 generation, all 3 groups of F_2 animals showed considerable advantage from the 30th to the 50th days.

In this generation also no definite changes in development were observed. Of the 3 groups, thymus treated, spleen treated and non-treated, the spleen treated group showed a slight advantage over the others in the time of eruption of teeth, opening of eyes and vaginal opening. In all 3 of these phenomena the average day of occurrence was slightly earlier in the spleen group than in the other 2, the thymus group next in order and the non-treated group last. However, compared with the 6 stock control litters run at the same time, the experimental animals (thymus, spleen and non-treated) showed a slight advantage only in the time of eruption of teeth. The eyes opened, in all 3 experimental groups, at approximately the same time as in the stock controls, but vaginal opening in the 3 groups of experimental animals occurred somewhat later than in the controls.

Three of the original 6 lines of progeny came to an end in this generation. Eight females died before maturity. Of the 18 females which survived, 2 were apparently not fertile, 8 died under treatment during pregnancy, or delivered dead or non-viable young. All 5 female descendants of one line died during pregnancy, aborted, resorbed their pregnancies, or delivered dead fetuses. As a result of these calamities only 8 F_2 females produced litters. Two stock males impregnated all of these females. Two females received thymus treatment during pregnancy, one received spleen treatment and 5 were not treated.

F_3 generation. Sixty-two offspring comprised this generation. Of these, 44 were from non-treated mothers (the grandmothers also had received no treatment), 15 were from thymus treated mothers (3 generations of treatment) and 3 from spleen treated mothers (spleen treatment for 2 generations following initial thymus treatment). In order to reduce the disporportion between groups, only 3 litters of the non-treated group were followed. Since there were only 3 animals in the spleen group, the weight curve for this group was not considered. The non-treated animals of this generation showed a considerably greater rate of growth than the thymus animals. No marked differences in development between these 2 groups were found nor were there any significant differences from the development of the stock controls.

In this generation only 5 females of the thymus treated group survived to maturity. Of these, 3 were not fertile. The 2 remaining females were both descendants of one of the original parents. Both were mated with the same stock male and were treated with thymus throughout pregnancy. The one female of the spleen group which survived to maturity became pregnant, but since sperm were not observed in the smear, treatment was not instituted. When the pregnancy was discovered it

was considered too late to begin treatment, therefore the spleen experiment was naturally terminated at this point.

Because of the drastic reduction in the number of experimental animals and the lack of definite effects resulting from treatment, it was decided to discontinue the experiment. However, since 3 F_3 female descendants of one of the original progenitors were pregnant at this time and 2 of them were being treated, it was decided to include the 4th generation offspring obtained from them in order to complete the experiment.

F_4 generation. When the 2 thymus treated F_3 females delivered it was noted that the average birth weight of the young (6.2 gm.) was well above the stock control average (5.3 gm.) and also greater than the average birth weight obtained in any

TABLE 1

	Stock cont.	F_1 Th ♀	F_2 Th ♀	F_2 Spl. ♀	F_2 No ♀	F_3 Th ♀	F_3 No ♀	F_4 Th ♀	F_4 No ♀
Mean birth wt.	5.3	5.5	5.7	5.6	5.3	5.1	5.9	6.2	6.0
Mean wt. at 10 days	15.0	18.2	15.8	16.4	14.5	14.5	17.1	19.0	20.0
Mean wt. at weaning (21 days)	29.9	34.5	31.4	33.4	31.2	29.1	34.7	39.6	39.2
Mean wt. at 50 days	107.3	122.0	116.8	130.0	124.4	101.8	121.4	138.6	142.2
Ave. day—eruption of teeth	10.9 (9–13)	10.4 (9–11)	9.8 (9–12)	9.4 (8–11)	10.1 (8–11)	9.6 (8–13)	9.8 (9–11)	9.5 (8–11)	10.7 (10–12)
Ave. day—opening of eyes	15.9 (13–18)	15.7 (14–17)	16.0 (14–18)	15.7 (15–17)	16.2 (15–18)	15.7 (15–17)	15.1 (13–16)	14.8 (14–16)	15.7 (15–16)
Ave. day—vaginal opening	50.6 (38–59)	51.2 (30–68)	55.0 (45–68)	52.7 (48–56)	55.1 (45–63)	53.4 (50–57)	56.7 (49–69)	52.2 (46–60)	55.6 (47–73)
No. of animals	92	27	34	12	15	14[1]	18	12	6
No. of litters	17	6	6	2	3	2[1]	3	2	1

[1] 2 extra animals marked and placed with foster mother.

other generation, treated or non-treated. This initial superiority in weight was maintained throughout the period of observation (table 1). These data on the F_4 generation compare favorably with the growth data presented by Einhorn and Rowntree (5) for their 4th generation thymus implanted animals. The average birth weight of their 4th generation animals was 6% above that of their stock controls, ours was 17% above control level; at 10 days their animals were 20% above the controls, ours were 27% above. At 50 days theirs were 8% above control level, whereas ours were 29% above. These results, without further analysis, appeared to be significant. However, when these F_4 thymus animals were compared with the untreated 4th generation litter, the results were not so striking. The antecedents of this litter had received no treatment for 2 generations, although the great, grandparent (F_1) had received thymus treatment during pregnancy. These animals, with an average birth weight of 6.0 gm., showed an even greater acceleration of the growth rate than the

F_4 thymus animals. This comparison, however, was not strictly valid inasmuch as this litter was the second litter born to the non-treated F_3 female. Unfortunately the entire first litter died soon after birth and, in order to have some check on the F_4 thymus animals, the mother was immediately remated. This procedure of remating had never been used before since it was felt that first litters should be used throughout for the sake of uniformity. It is impossible to say whether or not this factor influenced the growth rate of this particular litter. The stock control group (92 animals) with which they were compared had been selected at random and undoubtedly contained first, second and even later litters.

As an additional interesting observation, the birth weights of the two F_4 thymus litters (5.8 and 6.5 gm., respectively) and the one F_4 non-treated litter (6.0 gm.) were compared with normal birth weights for our colony. Of 586 consecutive normal deliveries of stock animals, 35 litters (6%) had average birth weights of 6.5 to 7.7 gm. This would indicate that the F_4 animals were not supernormal with respect to birth weight. However, compared with 5 control litters which had average birth

TABLE 2. DESCENDANTS OF TH I_0

Generation	F_1	F_2	F_3			F_4			Stock cont.	Super-av. stock
Treatment during embr. period	Th	Th	Th	Spl	None	Th	Th	None		
Av. birth wt.	5.0	5.8	5.5	5.7	6.2	5.8	6.5	6.0	5.3	5.9
Av. wt. at 10 days	13.0	16.5	15.0	13.3	14.5	18.5	19.5	20.0	15.0	16.5
Av. wt. at 21 days	29.3	26.5	25.1	27.3	33.0	37.7	41.5	39.2	29.9	32.1
Av. wt. at 50 days	124.0	93.2	95.3	100.3	124.3	128.7	148.5	142.2	107.3	114.1

weights of 5.8 to 6.3 gm., the F_4 animals (both treated and non-treated) tended to have a higher average weight at 50 days. The average weights of the 2 F_4 thymus treated litters at this age were respectively 13 and 30% greater than these 5 control litters. The non-treated F_4 litter averaged 33% higher. The F_4 animals therefore, not only maintained their initial superiority over the average stock controls, but also presented a higher degree of acceleration in their growth rate than the 5 stock litters which were above average in birth weight and growth rate. It is unfortunate that data concerning the growth rate of the stock animals of superior birth weight (6.5 to 7.7 gm.) cannot be presented for comparison.

Since all 3 F_4 litters were descendants of one F_0 animal, the possibility of an inherent tendency to a greater growth rate was investigated. The weight data on the progeny of the original mother (Th I) were isolated for each generation and compared with the data on the stock controls (table 2). No consistent and significant deviations from the controls were found in the thymus and spleen animals of the first, second and third generations, the general tendency was even toward a sub-control growth rate in these 3 generations. The non-treated animals of the third generation, however, showed a greater average birth weight and growth rate than the treated animals of the third generation and showed the same average birth weight, although not as great a growth rate as the 4th generation thymus animals. In view of the fact that the non-treated animals of both the 3rd and 4th generations showed some superiority over the stock controls, the apparently accelerated growth rate of the 4th generation thymus animals, in spite of the conformity with the results of Einhorn and Rowntree (5), cannot be considered a result of thymus treatment.

In the matter of development, the F_4 thymus animals showed no significant advance over the non-treated F_4 animals. However, the average times of eruption of the teeth and opening of the eyes were slightly earlier than in the non-treated animals. The average time of opening of the eyes (14.8 days) was the earliest found in any generation including the stock controls, although the average time of opening was only one day in advance of the stock control average. These results are not in accord with the results of Einhorn and Rowntree in their 4th generation implanted animals. In their animals the teeth erupted, on an average, 3 days in advance of the stock controls and the eyes opened 5.4 days earlier than in the controls. This represents an unusual precocity in development which was not obtained with our experimental method. It should be remembered that the developmental precocity and acceleration in growth obtained by the Rowntree group with daily administration of

TABLE 3. SUCCESSIVE LITTERS OF Th II_0—ALTERNATING B_1 AND NON-B_1

Litter	1	2	3	4	5	6	7
	Th B_1		Th B_1		Th B_1		Th B_1
Birth wt.	6.3	5.3	6.0	6.6	5.1	6.0	6.0
Av. wt.—10 days	19.7	13.3	19.6	18.1	19.6	19.9	21.5
Av. wt.—21 days	38.0	33.7	38.4	35.8	37.3	45.3	40.7
Av. wt.—50 days	118.7	106.0	120.4	132.8	115.1	143.5	148.4
Av. day—eruption of teeth	9.7 (9–10)	11.7 (11–12)	10.5 (10–11)	9.3 (8–10)	9.6 (9–10)	11.4 (11–12)	13.7 (13–14)
Av. day—opening of eyes	15.0 (15)	17.0 (17)	16.2 (14–16)	15.8 (15–16)	14.3 (13–16)	14.9 (14–17)	16.4 (16–17)
Av. day—vaginal opening	48.5 (45–52)	61.0 (61)	49.8 (44–56)	50.0 (50)	49.6 (43–57)	49.0 (49)	57.0 (57)
No. of animals	3	3	10	9	11	7	3
Date of delivery	4-1-39	5-27-39	7-21-39	9-24-39	11-16-39	1-18-40	3-2-40

thymus extract was even greater than with weekly implants of thymus. It would be expected, therefore, that the effects of treatment equivalent to the implantation of 6 thymuses weekly would also be greater than those obtained with implantation of 1 thymus weekly. This expectation has not been realized by our experiment.

A subsidiary experiment was carried on in which successive litters from one of the original females (TH II) were studied. This female was mated immediately after each weaning and thus delivered 7 litters. Thymus treatment was administered only during alternate pregnancies. The data on growth rate and development (table 3) showed no consistent variation between the thymus treated and the non-treated litters. In the 3rd, 4th, 5th and 6th litters, the number of offspring exceeded 6. The extra animals of each litter were marked and placed with foster mothers in litters also kept at a total of 6.

The 10 offspring of the 3rd litter (thymus treatment) were divided into two groups of 5 each. One group, with an added animal, was kept with the natural mother and treatment of the mother was continued throughout the lactation period (21 days). The other group of 5, with an added animal, was given to a foster mother. The growth curves for the 2 groups were practically identical throughout the 50-day observation period.

SUMMARY

Treatment of pregnant rats through 4 successive generations with fresh rat thymus failed to produce a significant acceleration of growth and developmental rates in the offspring. These negative results, however, cannot be considered as directly opposed to the positive results obtained by Einhorn and Rowntree since the procedures in the 2 cases were not identical. They implanted whole thymuses, one each week, beginning at 30 days and continuing for 12 weeks. In the present study, treatment was administered 6 times per week but only during pregnancy. They also presented data on more than one litter obtained from a single female, whereas our data was confined to first litters. They used litter-mate matings and we used stock males for matings except in a few cases of the first generation. We also reduced all litters, both experimental and control, to a total of 6 siblings in a further attempt at standardization. It is questionable, therefore, whether the lack of effect on growth rate in our experiment is due to inadequate treatment or whether the more rigid standardization of procedure eliminated a false effect due to other factors such as selective breeding or correlation between litter size and available nutriment per animal. The precocity evident in the early eruption of teeth, opening of eyes and vaginae which they found in their experimental animals has not been equalled in the experimental animals of this study. This would indicate that, if thymus treatment is the factor responsible for their developmental acceleration, treatment must be continued beyond the embryonic period.

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PERMANENT AFTER-EFFECTS FOLLOWING MASCULINIZATION OF THE INFANTILE FEMALE RAT[1]

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AN EARLIER REPORT (1) described masculinization of infantile female rats as a result of daily injections of anterior pituitary-like substance. Treatment was started at 6 days of age and the effect appeared within 3 weeks. Some of the rats that had been treated during infancy were observed for nearly a year after the cessation of treatment. Similar observations have been made on rats treated with testosterone propionate. The after effects of masculinization by either testosterone or anterior pituitary-like substance (2) are of sufficient interest to be reported in greater detail in this paper and to give some experimental evidence concerning the mechanism by which these effects are produced.

After Effects of Masculinization

Seven rats from a group that had been given 2 R.U. of antuitrin-S daily from the 6th until the 24th day of age still survived at 7 months of age. Daily vaginal smears taken during the 8th, 9th and 10th months revealed that all of these rats were in continuous vaginal estrus. This persistent estrus was also present in 5 rats that had been treated with testosterone propionate during the same period of time. Since vaginal smears had not been taken in the 2 months following cessation of testosterone treatment, a second experiment was started to determine how soon after the cessation of treatment the continuous estrous condition becomes established. Seventeen rats were each given 0.25 mg. of testosterone propionate on alternate days from the 6th until the 38th day of age (total dose, 3.5. mg.). The vaginal smears were diestrous for 2 weeks after cessation of treatment, but during the 3rd week all of the 17 rats came into a continuous vaginal estrus. After a month of continuous estrus a virulent organism produced tubal abscesses in 14 of the 17 rats. All of the rats were killed during the next 3 weeks. The infection was unilateral in 10 and bilateral in 4 of the animals. The experiment was repeated a second time with plans to take the vaginal smears by a more aseptic technic to avoid spreading infection from one rat to another. Sixteen rats were given 0.25 mg. of testosterone propionate on alternate days for 9 injections. Unfortunately the rats furnished for this experiment must have been about 4 days old at the beginning of the experiment since the development of the lower vagina was inhibited in 13 of the 16 rats treated. Only after surgical or mechanical dilatation was it possible to take the vaginal smears on this group.[2] At 6 months of age all 7 survivors were in continuous vaginal estrus. Three of this group of rats developed tubal abscesses during the 5th month.

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[1] This study was made possible by a grant from the Horace H. Rackham School of Graduate Studies to the Department of Obstetrics and Gynecology.
[2] Vaginal patency had occurred spontaneously by the 14th day of age in the original group. Apparently at 4 to 6 days of age there is a critical stage in the development of the lower vagina. Testosterone given at 6 days of age or later causes a precocious vaginal patency and at about 4 days of age will inhibit the formation of the lower vagina.

The end results of masculinization with either APL or testosterone are so similar that they can be discussed as an entity. Vaginal smears indicated a continuous follicular phase in the ovaries in nearly all of the treated rats. Histological studies of the ovaries showed that corpora lutea are consistently absent (fig. 8, 9, 10). These rats were placed in cages with males, and although mating occurred occasionally, it did not interrupt the continuous estrus by any diestrous phase comparable to a pseudopregnancy. Ovulation apparently did not occur, and these masculinized rats were sterile during the period of observation.

The high incidence of tubo-ovarian abscesses in these masculinized rats is of interest. The association of a prolonged estrous phase and an infection of the reproductive tract in this study is similar to that reported by Weinstein, Gardner and Allen (3). They reported a high incidence of pyometria in mice injected with estrogen for a long period of time. Gross pyometria has been observed only twice in our rats. The recent report of Shay, Gershon-Cohen, Paschkis and Fels (4) describes the production of ovarian tumors by treating infantile rats with testosterone. Our experience and their published photographs indicate they were describing tubo-ovarian abscesses rather than a neoplastic tumor as intimated in their article. They also describe this abscessed condition in one of their estrone treated rats. We have found several of these abscesses in our APL treated rats so that an infection seems consistently associated with a prolonged estrous state which may be induced by several methods of treatment. In the earlier stages of the infectious process only the fallopian tube is involved, and no change in the vaginal smears was noted. In some the infection advanced and involved the ovary, producing dense adhesions and in some cases completely destroying the ovaries. When this occurred the vaginal smears became dietrous. Figures 1 to 5 show several stages of this infectious process.

The continuous estrogenic stimulation was sufficient to produce marked vaginal and cervical hypertrophy in 2 rats in this study. Figure 6 shows the massive hypertrophy of the vagina of an APL treated rat that had been in continuous estrus for 6 months. The connective tissue elements seem more hyperplastic than the epithelium in this animal. Pfeiffer (5) has demonstrated similar pathological changes in the uterus and cervix of masculinized mice.

These observations extending over a 2-year period indicated that some permanent change must have been brought about by masculinization at this early age. In some ways this condition is similar to the free martin, an intersexual female twin calf which has been masculinized by the hormones from the male twin.

Mechanism of Persistent Estrus

The next phase of this study was to investigate the mechanism whereby masculinization of the infantile female rat leads to a permanent estrous state. Two possible explanations have been put to experimental test. It is well known that treatment with androgens produces a marked suppression of ovarian function, although the results of Mazer and Mazer (6) indicate that this suppression in adult rats is only temporary. The possibility remained that a profound deviation from normal development induced during infancy might produce irreparable ovarian damage so that only partial recovery would be possible. The second possibility was suggested by the report of Pfeiffer (7). He implanted testes into newborn female rats and induced masculinization by these testicular grafts. This led to a condition of continuous estrus which was maintained even after the grafts were excised. The results of a very complete series of experiments led Pfeiffer to postulate a sex difference in pituitary physiology. His theory explained the results on the basis that the pituitary of the newborn is undifferentiated. After the pituitary has differentiated in the presence of

the testicular hormone it liberates only the follicle stimulating hormone. After it has differentiated in the presence of ovarian hormone, it is capable of liberating both the follicle stimulating and the luteinizing hormones. Furthermore, once it has differentiated, its sex type cannot be altered. The work of Pfeiffer so closely parallels that reported in the first part of this paper that his hypothesis seemed worthy of further consideration.

The method of procedure chosen for this study was designed to test both of the foregoing theoretical possibilities. A reciprocal exchange of ovaries from a masculinized and a normal rat should test both the ovary and the physiological condition of

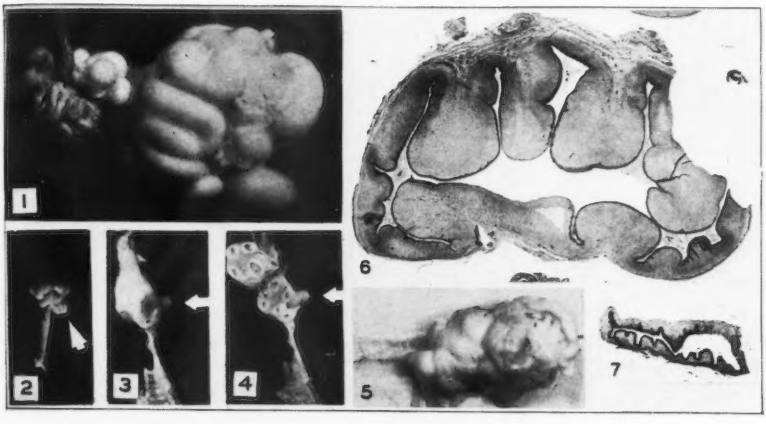

Fig. 1. Early stage of pyosalpinx showing the distended coils of the oviduct as contrasted with the tube and ovary of a normal rat. ×3. Fig. 2. Early stage of tubal infection. Ovary indicated by arrow. Fig. 3. Later stage of pyosalpinx with some adhesions. The ovary still visible is marked by the arrow. Fig. 4. Same as figure 3; cut to show the distended coils of the oviduct and local tissue reaction. Fig. 5. Large tubal abscess with many abdominal adhesions. Left ovary completely lost and undoubtedly destroyed by this infectious process. Right ovary in figure 8. (Figs. 2, 3, 4, 5, three-fifths natural size.) Fig. 6. Cross section of vagina of *rat* 27. Continuous vaginal estrus for past six months. See ovary in figure 9. ×3. Fig. 7. Cross section of vagina of normal mature rat. ×3.

the host. If the ovaries had been severely damaged by the masculinizing treatment, they should not function normally when implanted into a normal rat. If the masculinization had produced a change in the pituitary, as postulated by Pfeiffer, the ovaries taken from a masculinized female should recover and function more normally in a normal host. Furthermore normal ovaries implanted into a treated rat would show only follicular stimulation.

The method of Hill (8) for transplanting ovaries into the ear was used in this study. It afforded visible evidence of the condition of the graft and transillumination readily shows the presence of graafian follicles, cystic follicles, or corpora lutea. Seven rats were masculinized by 10 R.U. of antuitrin-S given daily from the 5th until the 28th day of age. Four of these were subjected to bilateral oöphorectomy on the 37th day of age. At the same time an ovary from an untreated control of the same age was put into each ear. The ovaries from each antuitrin-S treated rat were placed in the ears of the untreated controls. The implants failed to take in one untreated host and one ovary failed to vascularize in the ear of one of the APL treated rats. Ten rats were injected with 0.25 mg. of testosterone propionate on alternate days from the 5th until the 23rd day of age. On the 32nd day of age 5 of these were bilaterally ovariectomized.

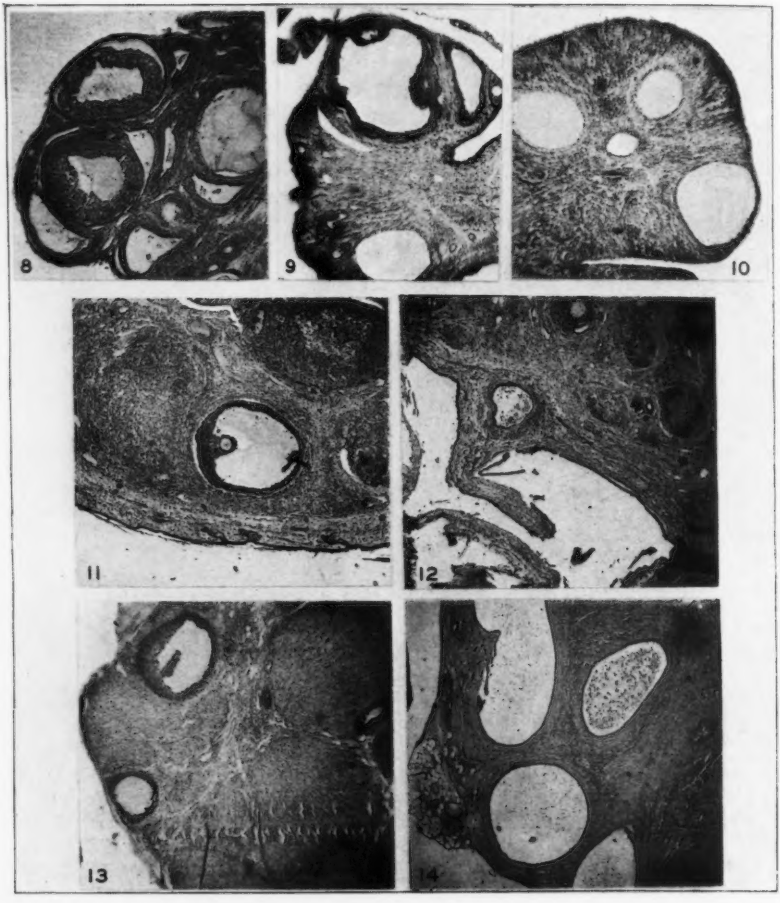

Fig. 8. RIGHT OVARY FROM *rat 51* TREATED WITH 0.25 mg. TESTOSTERONE ON ALTERNATE DAYS FROM THE 5TH TO 23RD DAY OF AGE. Continuous estrus from 60th to 104th day of age, when killed. No evidence of any corpora lutea. Large tubal abscess present on the left. See figure 5. Fig. 9. OVARY FROM *rat* 27. Had received 2 R.U. APL daily from 6th to 25th day of age. Continuous estrous smears for 5 months. When killed at 7 months of age no luteal tissue was present. Fig. 10. OVARY FROM *rat* 28. Same treatment as *rat* 27. See figure 9. Killed at 1 year of age after 10 months continuous vaginal estrus. Fig. 11. OVARIAN IMPLANT FROM TESTOSTERONE TREATED *rat* 10 IN THE EAR OF *rat* 1, PREVIOUSLY UNTREATED. Normal follicles and corpora lutea are present in this graft. Fig. 12. OVARIAN IMPLANT FROM UNTREATED *rat* 1 IN THE EAR OF TESTOSTERONE TREATED *rat* 10. Note the cystic follicles. No luteal tissue present. Fig. 13. OVARIAN IMPLANT FROM APL TREATED *rat* 32 IN THE EAR OF A PREVIOUSLY UNTREATED *rat* 22. Normal follicles and corpora lutea present. Fig. 14. OVARIAN IMPLANT FROM UNTREATED *rat* 22 IN THE EAR OF APL TREATED *rat* 32. Many cystic follicles and no corpora lutea.

Four of these received an ovary from an untreated control in each ear, while the 5th rat received the implants in the neck near the jugular vein. The ovaries from the 5 testosterone treated rats were implanted into the ears of 4 untreated controls and into the neck of the fifth. The implants failed to take in one treated and one control rat. These 2 had been mutual donors so there may have been some genetic incompatability.

Daily vaginal smears were taken starting 6 weeks after the ovaries had been transplanted into the ears. The 3 normal rats in which the grafts from APL treated rats became vascularized had a normal estrous rhythm. After 6 weeks observation of this normal rhythm a single ovarian implant was removed from each of 2 rats, and within 4 days the smears became continuously estrous. The ovaries removed before the onset of estrus contained corpora lutea (fig. 11). Three of the 4 APL treated rats that received normal ovaries had continuous or prolonged estrous smears and the implants were devoid of corpora lutea (fig. 12).

Vaginal smears revealed normal estrous rhythm in 2 of the 4 normal rats that had received ovaries from the testosterone treated rats. These 2 were killed after 6 weeks of vaginal smear observation and histological sections revealed that corpora lutea were present in the grafts (fig. 13). Continuous estrous was present in a 3rd rat in which only one ovarian graft became functional, and a continuous diestrus was noted in the 4th rat, in which the grafts resolved after 6 weeks.

The inhibition of the lower vagina in the testosterone rats made vaginal smear studies in this group very difficult. These rats had received ovarian implants from normal donors, and in no case was normal estrous rhythm noted, but a prolonged estrus was common. The sectioned ovarian implants had no evidence of any corpora lutea (fig. 14).

The most significant finding was that the ovaries taken from the testosterone and antuitrin-S treated rats vascularized and formed follicles and corpora lutea when implanted into normal rats. These ovaries would not have formed corpora lutea as long as they remained in the masculinized donor (fig. 8, 9, 10).

Of almost equal significance is the fact that normal ovaries implanted into masculinized females (testosterone and antuitrin-S treated rats) were characterized by the presence of follicles but never any corpora lutea. Pfeiffer's concept of a masculinized pituitary secreting only the follicle stimulating hormone would explain this situation.

Pituitary implants have not yielded sufficiently normal results in the experience of others to warrant an attempt to further this investigation by exchange of pituitary glands instead of ovaries. However, the results obtained here can be interpreted as further evidence in favor of Pfeiffer's hypothesis of a sexual difference in pituitary function. This is further substantiated by the fact that injection of human pregnancy urine, a luteinizing factor, will produce luteinization of the ovaries of these masculinized females.

If any clinical interpretations are to be drawn from this study, it would indicate that caution should be exercised in the use of testosterone in certain cases. The menorrhagia or functional bleeding of the young woman is associated with a continuous follicular phase in the ovary. If, by chance, her pituitary should be like that of a rat and has not differentiated sufficiently to produce luteinizing hormone, treatment with an androgen would inhibit the differentiation and tend to make the condition permanent. Estrogen therapy is not only effective in these cases of function bleeding but is a much more rational type of therapy, especially in view of Pfeiffer's theory. The latent after effects of androgenic therapy may be of more clinical importance than an apparent immediate benefit.

SUMMARY

The after effects of masculinization of the infantile rat by treatment with either APL or testosterone are characterized by a continuous vaginal estrus. The ovaries contain follicles but no corpora lutea, and mating attempts indicate that the rats are permanently sterile. Tubal abscesses occur frequently. This method of producing a permanent estrous state should be an easy and economical way of producing most of

the experimental pathology (mammary carcinoma, etc.) that has been achieved by prolonged estrogenic treatment.

The mechanism whereby treatment with androgens produces a continuous estrus as an after effect is not permanent ovarian damage. Ovaries from masculinized rats recover sufficiently to form corpora lutea when implanted into normal rats, and normal ovaries implanted in the masculinized animals failed to form corpora lutea. The results indicate that the permanent change in the fundamental physiology is probably in the anterior pituitary. The concept of Pfeiffer on a sexual difference in pituitary function is adequate to explain the results obtained.

The author is indebted to Miss Helen McRae for technical assistance which made it possible to ovariectomize 2 rats (one treated and one control) simultaneously so that their ovaries could be exchanged and immediately implanted. The testosterone propionate (neohombreol) was generously supplied by Roche Organon. The anterior pituitary-like substance, antuitrin-S, was furnished by Parke, Davis & Company.

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THE ACTION OF TESTOSTERONE PROPIONATE ON EXPERIMENTAL MENSTRUATION IN THE MONKEY[1]

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CLINICAL OBSERVATIONS have shown that when masculinizing changes begin in girls, as in virilism, menstruation becomes irregular, scanty or stops for long periods. This has led to investigations of the possible rôle of androgens in the menstrual cycle. It is possible experimentally to inhibit the rhythmic occurrence of menstruation by administration of androgen in the intact monkey (1, 2) and in women (3, 4). The uterine bleeding which follows the cessation of adequate estrogenic stimulation in monkeys (5) has likewise been inhibited by the subsequent administration of androgen (2). Relatively high doses have been used to accomplish this androgenic inhibition.

Testosterone has several biological reactions fairly similar to those of progesterone. One of these is the inhibition of experimental menstrual bleeding in spayed monkeys subject to estrogen-deprivation (6). In spite of the observation that these two hormones exert a different histological reaction on the endometrium (7), the actual mechanism whereby these hormones inhibit uterine bleeding still remains obscure. Therefore, an insight into the mode of action of these hormones on the endometrium may be reached if significant differences can be shown with respect to their effect on menstrual bleeding.

Since it has been shown that androgens are consistently present in the female as well as the male (8), the possibility arises that androgens may normally participate in certain of the processes serving to govern the menstrual cycle in the normal female. This possibility as well as the current clinical use of androgens in a variety of menstrual and other gynecologic disorders (9–14) warrants further study of the action of androgens on the menstrual process. For these reasons the following two questions have been studied in this investigation: (*a*) what is the minimal effective dose of androgen required to inhibit bleeding occurring after cessation of estrogenic treatment? and (*b*) does estrogen prevent uterine bleeding following injections of combinations of estrogens and androgens? The attempt to answer these questions utilizes experimental menses induced by estrogen-deprivation in ovariectomized monkeys as end point.

MATERIALS AND METHODS

Eleven mature spayed monkeys (*Macaca mulatta*), weighing from 3.6 to 5 kg. were used in the present study, several of them repeatedly. All injections were given intramuscularly deep into the buttocks. The estrogen employed was estrone,[2] which was administered as a constant daily dose of 50 micrograms dissolved in 0.25 cc. of sesame oil. This amount of hormone considerably exceeds the daily threshold level of estrogen necessary to induce uterine bleeding following the cessation of a 10-day

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[1] This study was aided by grants from the Committee for Research in Problems of Sex of the National Research Council and the International Cancer Research Foundation.
[2] Estrone was theelin furnished through the courtesy of Parke, Davis & Co.

period of treatment (15, 16) and will be referred to as the priming dose of estrogen. Testosterone propionate[3] was used in daily doses of 0.5 to 4 mg., routinely dissolved in 1 cc. of oil. In the experiments designed to study the minimal effective dose, peanut oil served as the vehicle. In the instances when testosterone propionate was injected simultaneously with estrone either peanut or sesame oil was used.

Daily observations were made on the color and swelling of the "sexual skin,"

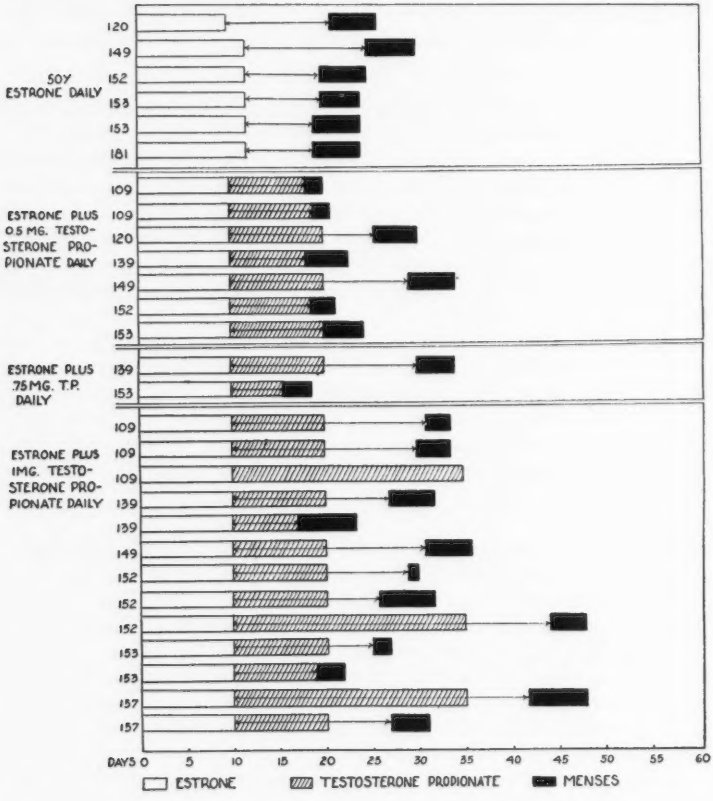

Fig. 1.

thickness of the vaginal epithelium and the size of the clitoris. A small speculum was used in the daily vaginal examinations for detection of the earliest evidence of uterine bleeding.

OBSERVATIONS

Action of testosterone propionate following estrogen priming. Daily injections of 0.5 mg. of testosterone propionate were made following preliminary priming treatment with estrone. Five of the 7 experiments in this series showed no apparent effect of the androgen at this level (fig. 1, second series). Endometrial bleeding occurred from

[3] Testosterone propionate was furnished through the courtesy of the Ciba Pharmaceutical Products, Inc., under the trade name perandren.

7 to 9 days after estrone withdrawal and during the period of injection of testosterone propionate. These results are comparable to those found with estrogen alone after discontinuance of the priming dose (5). Compare first and second series in figure 1. In 2 animals uterine bleeding did not occur during the 10-day period of injections of testosterone propionate but was delayed until the 15th and 19th day following estrone withdrawal or 5 and 9 days after the cessation of androgenic injections. The 2 animals (120 and 149) weighed 3.6 and 3.7 kg. respectively or approximately one

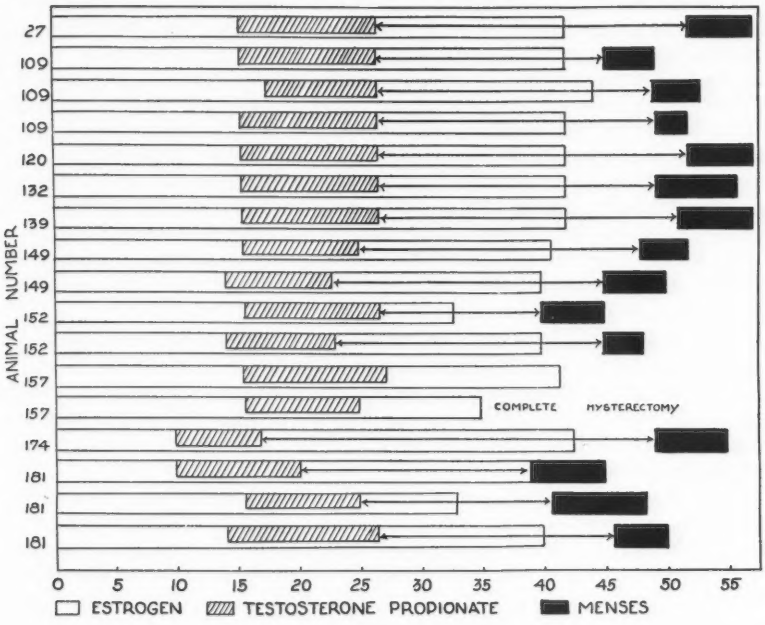

Fig. 2.

kilogram less than the other animals in this series. Therefore 0.5 mg. may have been the threshold level for these animals at this time. However, both these animals also showed a delayed onset of bleeding when treated with a priming dose of estrone alone.

Two animals were treated wtih 0.75 mg. of testosterone propionate daily following a similar preliminary treatment with estrone. In one instance, no effect of the androgen was noted as bleeding occurred on the 5th day following withdrawal of the estrogenic treatment. The other animal did not bleed during the 10 days testosterone propionate was injected, but bleeding occurred 20 days following the withdrawal of estrone. Compare these results with reactions of the same animals in the second series, figure 1.

Injection of 1 mg. of testosterone propionate daily for 10 days definitely inhibited the onset of uterine bleeding in 8 experiments. Bleeding did not occur until 15 to 21 days after the cessation of treatment with estrogen (fig. 1, fourth series). Bleeding in the other 2 animals was not inhibited by this procedure, occurring on the 7th and 9th day of treatment with androgen. The onset of menstruation in both these animals (139 and 153) had been inhibited successfully in previous experiments with the same dose of androgen.

Bleeding did not appear during the course of treatment in 3 animals given 1 mg. of testosterone propionate daily for 25 days following initial priming with estrone. Two of these animals showed uterine bleeding on the 32nd and 34th day, respectively, after withdrawal of estrogenic treatment. With the technic of intravaginal inspection uterine bleeding was not observed at any time in the third animal, although other signs of hormonal activity (e.g., sex skin reaction) had been present.

Action of testosterone propionate combined with a continuous estrogenic treatment. After administration of the standard daily dose of estrone to 17 animals for a period of 10 to 17 days, 2 mg. or 4 mg. of testosterone propionate were given daily for periods of 8 to 14 days in addition to continuation of the injections of estrone (fig. 2). The injections of estrone were further continued for 6 to 26 days following the end of the androgenic injections. With one exception, uterine bleeding did not occur throughout the period of estrone injections, but occurred only at the expected time following cessation of the estrogenic injections. If progesterone, instead of testosterone, had been injected bleeding would probably have occurred within a few days after stopping progesterone, in spite of continuance of estrogen (18). Bleeding occurred in one animal (*181*) on the 38th day of injection with estrone and 19 days after the last dose of testosterone propionate. Occasional reactions of this character are known to occur, however, in animals treated with a similar daily dose of estrogen over a prolonged period (16).

Time of onset and duration of uterine bleeding following cessation of treatment with an effective level of testosterone propionate. In 13 experiments, noted above, in which testosterone propionate definitely delayed the onset of uterine bleeding, blood was noted in the vagina beginning 5 to 11 days (average, 8 days) after the last injection of testosterone propionate. The duration of such bleeding ranged from 2 to 8 days, with an average of 5 days

Other actions of testosterone propionate in the female monkey. Daily observations of the color and edema of the sex skin revealed markedly individual responses to the injections of the hormones used in these experiments. These factors, therefore, were not considered as reliable criteria in following the response of the animals to the hormonal treatment. In general, the coloration of the sex skin was maintained upon adequate treatment with testosterone propionate.

The repeated series of injections of testosterone propionate over the long experimental period to which some of these animals were subjected, frequently caused a marked enlargement of the clitoris which did not regress completely when androgenic treatment was discontinued.

DISCUSSION

Previous investigations (2, 7) have reported the inhibition of estrogen-deprivation bleeding with a daily dose of 5 mg. of androgen. Our results indicated this to be above the minimal effective dose. Bleeding was successfully delayed in 11 of the 13 experiments in which 1.0 mg. of testosterone propionate was injected daily for 10 and 25 days following a preliminary estrogenic priming. Decrease of the daily dose of testosterone propionate to 0.5 mg. showed this to be too low a level for bleeding occurred in 5 of the 7 experiments within the range of latency attributed to the action of estrogen alone (5). These results indicate that under the conditions of these experiments a daily threshold of 1 mg. of testosterone propionate approached the minimal effective dose necessary to prevent the onset of estrogen-deprivation bleeding. Failure to inhibit bleeding with the 1 mg. dose of testosterone propionate daily in 2 animals, although a similar dose had previously prevented bleeding, must be explained at present under the vague term, biological variation.

Under similar experimental conditions, 0.5 mg. of progesterone has been stated to be the minimal effective dose necessary for inhibition of estrogen-deprivation bleeding (17). In view of this observation, our results would indicate that, by weight, about twice as much testosterone propionate as progesterone is required to inhibit the bleeding which follows cessation of injections of estrogen.

Our experiments (fig. 2) demonstrate uniformly that continued estrogen sustained the endometrium and prevented bleeding following treatment with testosterone propionate until such a time as bleeding could be attributed to the effects of the estrogen withdrawal alone. Cessation of progesterone injections, under similar experimental conditions, induced uterine bleeding in 3 to 5 days despite continued injections of previously adequate doses of estrogen (18).

SUMMARY

It had previously been shown that testosterone, as well as progesterone, would prevent the experimental menstruation which follows withdrawal of estrogenic stimulation of the uterus. We find that the minimal effective dose of testosterone propionate for postponing menstruation in monkeys after 50γ of estrogen daily for 10 days is approximately 1 mg.

The interval between cessation of an effective inhibiting dose of testosterone and the onset of bleeding is similar to that following withdrawal of estrogen; both are longer than that following withdrawal of progesterone as stressed by Engle.

In the monkey receiving continuous estrogenic treatment at the same dose level, it has been shown previously that after supplementary administration of progesterone bleeding occurs. In a similar experiment the use of testosterone instead of progesterone was not followed by bleeding. In addition to the different histologic response of the endometrium (7), there is, therefore, a different physiologic action of testosterone and progesterone.

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THE GROWTH DEPRESSING EFFECT OF LARGE DOSES OF TESTOSTERONE PROPIONATE IN THE CASTRATE ALBINO RAT

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IT HAS ALREADY BEEN SHOWN that 1 mg. testosterone propionate administered daily (except Sunday) from 26 to 80 days to normal male albino rats inhibits the growth of such animals (1). This dosage in the presence of the gonad was believed to be physiologically excessive for which reason it was considered to depress the pituitary growth (as well as sex) function. Since it had already been shown that castration in the male rat also resulted in growth depression (2, 3) it became interesting to study the effect of this dosage (1 mg.) in such castrate animals in which the production of androgens is naturally absent.

For this purpose 46 male albino rats of Wistar Institute strain were divided into 3 groups. Group 1 consisted of 16 untreated, unoperated controls; group 2, comprised 15 untreated castrate controls; while the third group contained 15 castrate (test) animals each of which received daily (except Sunday) intraperitoneal injections of 1 mg. testosterone propionate in sesame oil (perandren) from 26 to 80 days of age. All 3 groups were made up of respective litter-mate brothers. Castrations were carried out at 22 days of age. Initial, weekly and final body weights and final body lengths (taken from anus to the tip of the snout) were determined for each animal. Aside from injections given to the test group, all animals were treated in the usual manner previously described (1, 3). The data obtained from these observations were massed and treated statistically (4), and observed differences were considered as 'probably significant' only if 'significance ratios' were 3 or more.

The animals were killed on the 80th day by severing the carotid vessels while they were under the influence of ether anesthesia.

RESULTS

From table 1 it may be seen that initial body weights showed no significant differences between any of the 3 groups. After treatment, however, it may be observed that the castrate control (192.0 ± 3.9 gm.) was 25.9 ± 5.17 gm. lighter than the normal control (217.9 ± 3.4 gm.). This difference with its significance ratio of 5 was 'probably significant.' The final body weight of the castrate treated animals (181.1 ± 2.7 gm.) was 36.8 ± 4.33 gm. lighter than the normal controls. This difference with its significance ratio of 8.5 showed a still greater probability of significance than that observed between the two sets of controls.

The final body length of the castrate untreated animals (194.2 ± 1.42 mm.) was 10.8 ± 1.9 mm. less than that of the normal controls (205 ± 1.2 mm.). This difference leads to a significance ratio of 5.7. The final body length of the castrate treated animals (191.5 ± 0.94 mm.) was observed to be 13.5 ± 1.5 mm. less than that of the normal controls. The significance ratio of this difference is 9.0. The growth curves of the 3 groups of animals based on their average weekly weights are shown in figure 1.

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DISCUSSION

It will be observed that initial body lengths were not determined. This omission was deemed permissible since such measurements taken on squirming animals are never truly accurate. Nor would the use of hypnotics to minimize squirming be quite acceptable since such treatment could conceivably affect the animal's future growth tendency. The high coefficient of correlation normally existing between body length and body weight in the albino rat (5) gives weight to the probability that

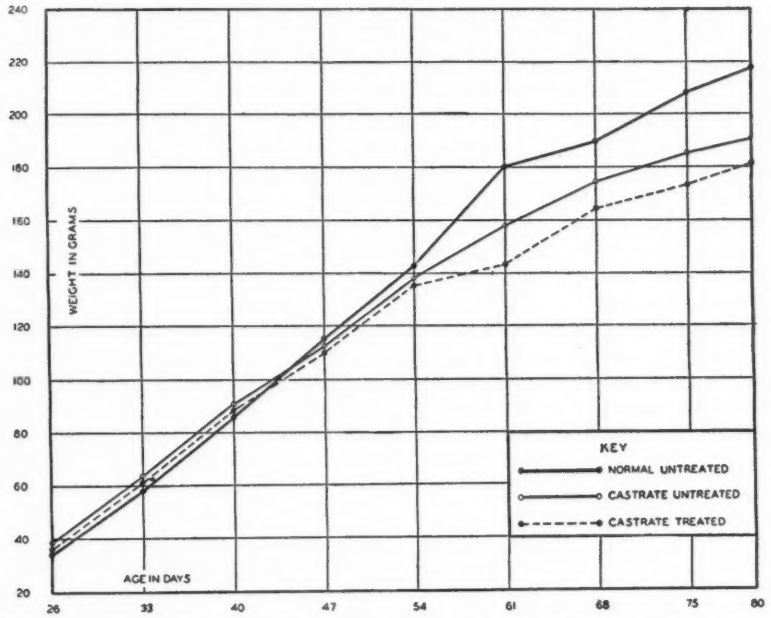

Fig. 1. Comparison of the mean body weights of castrated male rats treated with 1 mg. testosterone propionate daily, except Sunday, with normal and castrated untreated animals.

all animals were approximately the same length at the beginning of the experiment.

The diminished body weight and length in the untreated castrate rat has been previously reported (1–3, 6–9). One would ordinarily expect that after castration, replacement therapy with large doses of testosterone propionate would stimulate general somatic growth. As observed in these experiments however the growth of the castrate treated animals was still further depressed.

While it is not shown in table 1, the difference between the final body weight of the castrate treated and castrate untreated animals is 10.9 ± 4.6 gm. in favor of the untreated group. This leads to a 'significance ratio' of 2.4. Although this difference is not statistically significant it none the less indicates the trend of divergence in growth between these two sets of animals.

It appears therefore that the further growth depression in the treated castrates represents the summation of effect brought on by castration and pituitary suppression due to excessive sex hormonal administration (10–14). This effect stands in contrast to other observations in which appropriately smaller doses of testosterone propionate not only stimulate body growth in the normal animal (15) but actually counteract the general somatic growth depression induced by castration (16).

TABLE 1. COMPARISON OF INITIAL AND FINAL BODY WEIGHTS AND FINAL BODY LENGTHS OF CASTRATE UNTREATED AND CASTRATE TREATED RATS WITH RESPECTIVE FINDINGS IN NORMAL CONTROL ANIMALS. TREATMENT CONSISTED OF DAILY (EXCEPT SUNDAY) INTRAPERITONEAL INJECTIONS OF 1 MG. TESTSTERONE PROPIONATE FROM 26 TO 80 DAYS.

	At 26 days			At 80 days					
	Body wt.	Diff.	S.R.	Body wt.	Diff.	S.R.	Body length	Diff.	S.R.
	gm.			*gm.*			*mm.*		
Normal Control 16 animals	36.0±1.4			217.9±3.4			205.0±1.2		
		2.4±1.9	1.26		25.9±5.17	5.0		10.8±1.9	5.7
Castrate Untreated 15 animals	38.4±0.9			192.0±3.9			194.2±1.42		
		1.2±1.8	0.66		36.8±4.33	8.5		13.5±1.5	9.0
Castrate Treated	37.2±0.6			181.1±2.7			191.5±0.94		
15 animals									

SUMMARY

Testosterone propionate given in doses of 1 mg. daily (except Sunday) from 26 to 80 days, depressed the body length and weight of the castrate male albino rat to a still greater degree than that observed following castration alone. It is felt that this added depression represents a summation of the effects of castration plus the pituitary depression incident to overdosage with sex hormones. The added growth depression of large (1 mg.) doses of testosterone propionate in the castrate male albino rat stands in contrast to the replacement effect of appropriately smaller doses (50γ) of this same hormone.

The authors are grateful for the cooperation of the Ciba Pharmaceutical Products Company, Inc., for partially defraying the expense of this study and for furnishing the testosterone propionate (perandren) used.

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QUANTITATIVE RELATIONS OF A PROSTATIC COMPONENT (ACID PHOSPHATASE) OF HUMAN SEMINAL FLUID

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A SPECIFIC acid phosphatase with optimum activity in the approximate range pH 4.0 to 6.0 is present in high concentration in human prostate tissue (1, 2) and in the caudal lobe of the Rhesus prostate gland (3). The enzyme normally appears in the prostate gland after puberty (4) (precociously however in prostate glands of prepubertal Rhesus monkeys treated with testosterone propionate (3)) in amounts ranging from roughly 500 to 2000 U per gm. wet prostate tissue; phosphatase concentrations far greater than are found in any other tissue (4). Prostate acid phosphatase is secreted externally in the prostatic fluid[1] and constitutes a chemically identifiable prostatic component of the semen (1). Since other glands of the male genital tract secrete no significant acid phosphatase into the seminal fluid (1), it is possible to estimate the prostatic contribution to the semen by determining the acid phosphatase activity of whole and fractionated ejaculates. The results of such determinations are recorded here and serve to supplement similarly directed studies of the morphological (6) and chemical (7, 8) constituents of the seminal fluid.

METHODS

Fifty-seven specimens from 16 normal, young subjects were studied. Most of the specimens were centrifugates of ejaculates obtained for investigation of sperm metabolism (9) by Dr. John MacLeod, to whom we are greatly indebted. The supernatant of the centrifugates was diluted 1:10 with normal saline solution, then for optimal concentration, further diluted 500 to 4000 times, depending on the results of preliminary runs. Acid phosphatase activity was determined by an adaptation (10) of the King and Armstrong method for estimating alkaline phosphatase (11). Hydrolysis was conducted at pH 4.9, using citrate-HCl buffer, for ½ hour at 37° C. All determinations were made in duplicate, agreement between duplicates being within 5%. The results are expressed as units of acid phosphatase activity, a unit being defined as that degree of activity which at 37° C. will liberate 1 mg. of phenol in 1 hour from the specified monophenylphosphate substrate-citrate buffer solution at pH 4.9.

RESULTS

Specimens collected at approximately weekly intervals. The acid phosphatase activities of the seminal fluid of 10 normal subjects from whom at least 3 ejaculates were obtained are summarized in table 1. The values range from 540 to more than 4000 U per cc. seminal fluid and from 1800 to over 17,000 U in the whole specimen.

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[1] In the special case of subjects with prostatic carcinoma invading lymph or blood channels, prostate acid phosphatase escapes into the circulating fluids (5). Under these circumstances it becomes, in a sense, an internal secretion.

In different subjects in this normal series, the acid phosphatase levels varied from 700 ± 150 U to 3700 ± 500 U per cc. seminal fluid. The range of variation in the several ejaculates obtained from any one individual, however, was relatively small (table 1).

The total volume of the ejaculates varied from 1.2 to 5.4 cc., most of them falling within the range 2.0 to 3.5 cc. The volume of packed sperm in rapidly centrifuged specimens was small (about 0.02 cc.) and the acid phosphatase activity of spermatozoa per specimen was found to be low (about 0.4 U). The alkaline phosphatase activity (pH 9.0) was determined in 12 seminal fluids and found to vary from 0.1 to 1.0 mg. phenol liberated per cc. per hour.

TABLE 1. ACID PHOSPHATASE ACTIVITY OF SEMINAL FLUIDS OF 10 NORMAL SUBJECTS (RESULTS EXPRESSED IN U PER CC. SEMINAL FLUID)

Subject	Specimen No.					
	1	2	3	4	5	6
	U	U	U	U	U	U
F	2450	2350	3060	2470	2710	2750
K	1960	1950	2320	2300	1630	2915
R	810	600	790	1040	710	805
B	3200	3350	3290	3170	3160	
G	1360	1370	1510	1920		
C	840	540	810	780		
H	3200	4040	4220			
S	3450	2250	3610			
A	2230	2020	2630			
W	1220	1410	960			

Successive specimens obtained after brief intervals. A moderate decline in acid phosphatase values was noted in successive ejaculates obtained at hourly intervals in subject *L* (1130 U per cc., first seminal fluid; 880 U per cc. second seminal fluid) and in subject *O* (2550 U and 2180 U, respectively) whereas in subject *T* the second hourly specimen was higher than the first (1600 and 2600 U, respectively). No significant difference was noted between 8-hour interval specimens; for example, in subject *N* values of 1620 and 1890 U, respectively, were obtained.

Fractionated specimens. In 4 samples from one individual, the first, middle and last portion of each ejaculate was collected separately. The volumes and acid phosphatase activities of these fractions are recorded in table 2. It was found that prostate acid phosphatase is not uniformly distributed throughout the ejaculate since the last fraction (volume 0.7 to 0.85 cc.) contained comparatively little enzyme—not more than could be accounted for by the flushing out by some non-prostatic secretion of residual prostatic fluid in the urethra. The first cc. of seminal fluid, on the other hand, contained by far the highest concentration of acid phosphatase and therefore of prostatic secretion. The first 2 cc. of seminal fluid held most of the acid phosphatase present in the ejaculate, i. e., the prostate gland liberates its secretion into the seminal fluid early in the course of ejaculation and in the subject investigated the bulk of the prostatic fluid was contained in the first half of the ejaculate.

DISCUSSION

It would appear from the data in table 1 that normal subjects vary considerably with respect to the prostatic component of their semen, as in regard to other morphological and chemical characteristics of the spermatozoa and seminal fluid, but that the prostatic secretion per ejaculation of any one normal individual is maintained at a

fairly consistent level. It is not clear from our data whether the differences observed in various persons are due chiefly to variations in the amount of prostatic secretion or in the acid phosphatase content of normal prostatic fluids. This distinction could be made if prostatic fluids free of other secretions were available for analysis but attempts to obtain such material by prostatic massage failed.[2] However, in view of the known wide variation in the acid phosphatase content of prostate tissue of normal adult males, it is highly probable that the acid phosphatase content of the secretion of the prostate gland in different individuals varies considerably.

There has been much discussion as to whether the ejaculate in man is a haphazard mixture of the several glandular secretions of which it is composed or whether there

TABLE 2. ACID PHOSPHATASE ACTIVITY OF FIRST, MID- AND LAST PORTIONS OF EJACULATES COLLECTED FRACTIONALLY (4 EJACULATES FROM 1 PERSON)

Ejaculate No.	Fraction No.	Volume cc.	Units acid phosphatase activity in fraction	per cc. fraction
	1	0.85	2760	3250
1	2	1.40	1160	830
	3	0.85	300	350
	(total)	3.10	4220	
	1	0.90	2745	3050
2	2	3.20	4480	1400
	3	0.85	270	315
	(total)	4.95	7495	
	1	1.80	7200	3950
3	2	2.20	1600	730
	3	0.70	310	450
	(total)	4.70	9110	
4	1	1.20	2270	1890
	2	2.20	1380	630
	(total)	3.40	3650	

is an orderly, perhaps purposeful, sequence in the addition of each component. Microscopic studies of partitioned ejaculates by Broesike (6) favor the latter interpretation. Broesike distinguished 4 phases of ejaculation: (*a*) pre-ejaculatory emission of 1 or 2 clear, viscid drops representing the secretion of Cowper's or of urethral glands (we found these pre-ejaculatory drops to be virtually devoid of acid phosphatase); (*b*) prostate gland secretion, composing the first part of the ejaculate proper, which is almost free of spermatozoa; (*c*) mid-portion, containing the bulk of the sperm; (*d*) seminal vesicle secretion, composing most of the last part of the ejaculate. Scherstén's studies of the citrate content of fractionated ejaculates (8) and the analyses recorded here (table 2) support the view that the first portion of the ejaculate consists chiefly of prostate gland secretion.

Phosphoric esters (phosphocreatin, nucleic acid derivatives) are known to be present in seminal fluid and as the slightly acid pH of adult vaginal secretions offers a favorable medium, it is likely that prostate acid phosphatase is active in inseminated

[2] In subject R, for example, 3 specimens obtained by prostatic massage (with careful avoidance of the area proximal to the seminal vesicles) contained only 840, 755 and 612 U, respectively, of acid phosphatase per cc. prostatic fluid. It is clear from a comparison of these values with the results on ejaculates in this subject (table 1) that the prostatic fluid obtained by us was grossly contaminated with other secretions.

ejaculates. The physiological significance of such enzymic activity, i.e., this function of the prostate gland, is not known. The relation of erythrocyte phosphatase to glycolysis in shed blood suggests the possibility of an analogous relation between prostate acid phosphatase and glycolysis in semen. Since the metabolism of human spermatozoa is almost exclusively glycolytic (9), this possibility is consistent with the impression of many years standing that prostatic secretion favors spermatozoan motility. We have considered also the possibility that prostate acid phosphatase (by splitting off phosphoric acid) might be responsible in part for the precipitation of insoluble spermine phosphate in ejaculates allowed to stand. Experiments in which sodium fluoride was added to fresh seminal fluid to inhibit acid phosphatase activity (12) gave equivocal results however. With regard to the rôle of prostate acid phosphatase in male sterility, we examined samples from 24 sterile males. The semen of one subject contained only 33 U of acid phosphatase activity per cc. seminal fluid; the others gave results within normal limits.

SUMMARY

The acid phosphatase of seminal fluid is derived from the prostate gland and its quantitation affords a measure of the prostatic component of human semen.

The results of acid phosphatase determinations in 43 ejaculates obtained from 10 normal individuals are recorded. The values ranged from 700 ± 150 U to 3700 ± 500 U per cc. seminal fluid. The enzyme level in the several ejaculates of any one individual was relatively constant.

Acid phosphatase determinations in specimens collected fractionally indicated that the prostate gland liberates its secretion into the seminal fluid early in the process of ejaculation and that the first cc. of the ejaculate is composed largely of prostatic fluid.

The possible physiological significance of prostate acid phosphatase is considered.

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CUTANEOUS VASCULAR AND PIGMENTARY CHANGES IN CASTRATE AND EUNUCHOID MEN

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ONE OF THE OUTSTANDING CHARACTERISTICS of a human male castrate is the paleness of the skin. After treatment with androgenic hormone, however, the individual takes on a darker and more ruddy hue (1). This observation suggests that the skin of the castrate is deficient in melanin and blood, and that the androgenic hormone increases the content of these substances in the integument.

Recently the Hardy recording spectrophotometer has been used to measure objectively the pigments of the intact normal skin (2). This has led the present authors to collaborate in a spectrophotometric analysis of the skin of human male castrates and eunuchoids before, during, and after the cessation of treatment with crystalline androgens.

MATERIALS AND METHODS

The subjects forming the basis of this report include 4 castrate and 5 eunuchoid individuals. Three of the castrates (including one Negro patient) had undergone surgical removal of the testes; the fourth was castrated in an accident. The 5 eunuchoids include 3 men lacking sexual maturation, one hypogonad dwarf, and one unilaterally cryptorchid man whose normally descended testis had been surgically removed when he was 35 years of age, and whose cryptorchid testis is apparently practically functionless. The term eunuchoid is applied to individuals whose testes although present do not function adequately, so that normal levels of androgens are not maintained in bodily tissues and fluids.

In conjunction with Doctors R. I. Dorfman and H. Catchpole, biologic assay was carried out of the gonadal and gonadotropic substances in the urine of each of these patients. These determinations were performed before, during and after the withdrawal of, treatment. The methods of assay and the results are published in detail elsewhere (3, 4).

Testosterone propionate was used almost exclusively in the treatment of the patients. It was given in intramuscular injections, usually in a dose of 20 mg. in 1 cc. of peanut oil. The eunuchoid patient. J. M., received a single injection of 150 mg. of androsterone in peanut oil.[1]

The spectrophotometric measurements were made in a room equipped with an air-conditioning unit in which the temperature was maintained at 80° F. within a 5° range of fluctuation. The relative humidity was maintained at a level of 10 to 30%.

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[1] The androgens, testosterone propionate and androsterone, were furnished by the Ciba Pharmaceutical Products, Inc.

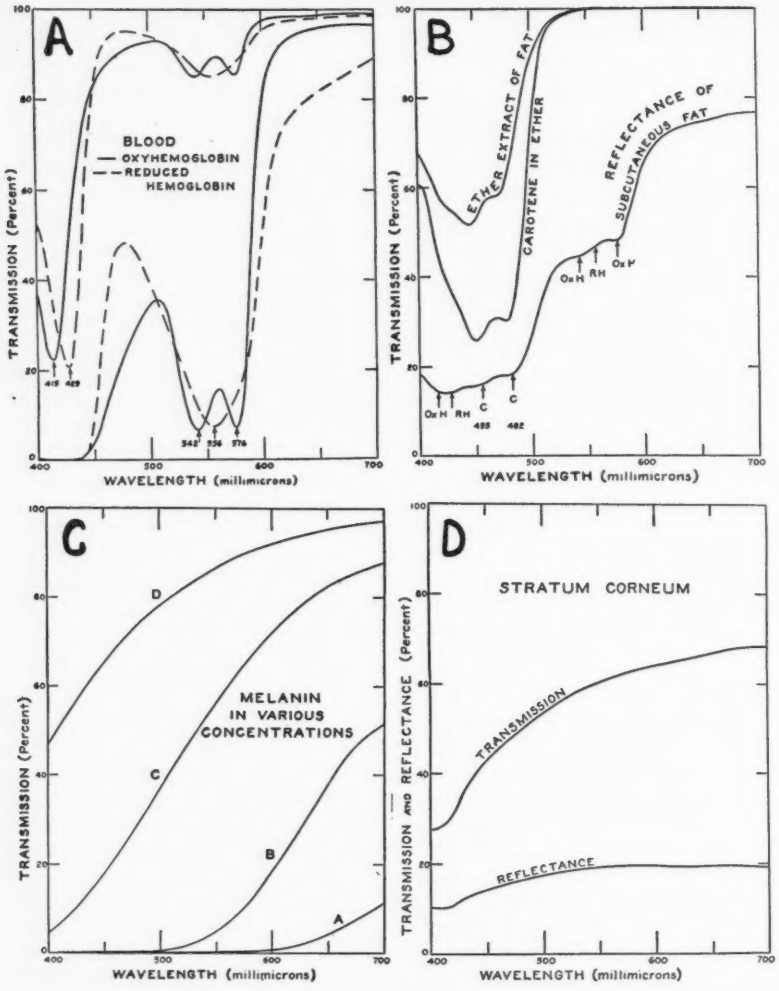

Fig. 1. Sepctrophotometric curves of the cutaneous pigments, (*Am. J. Anat.* 65: 1, 1939).

A. The transmission of reduced and oxyhemoglobin. In the lower curve the blood was diluted 1 to 100; in the upper curve it was diluted 1 to 1600. Oxyhemoglobin shows a pair of hemoglobin bands at 542 and 576 mμ. By contrast, in reduced hemoglobin these bands are replaced by a single broad band at 556 mμ. The general shape of the two curves is also of significance—the reduced form showing a higher reflectance in the violet and blue regions of the spectrum, but a decidedly lower reflectance in the orange and red.

B. The absorption of carotene with bands at 455 and 482 mμ in curves of subcutaneous fat and of known solutions of carotene. Ordinarily, in the intact skin the band at 455 mμ is obscured by the absorption of hemoglobin, and only that at 482 mμ is evident.

C. The transmission of melanin in 5% KOH solutions. There is a marked absorption in the violet end of the spectrum, although no demarcated bands are seen.

D. The transmission and reflectance of stratum corneum obtained from a cadaver's heel. Both curves show the general slope and the absorption band at 400 mμ, characteristic of melanoid.

The subjects rested for from 20 to 40 minutes before readings were begun. Areas of the body which remained uncovered by clothes, such as the face and hands, were measured last, to allow additional time for the circulation to reach stability. As far as possible, we avoided taking readings at night or soon after meals, since a peripheral vasodilatation may be present at these times.

A description of the Hardy recording spectrophotometer (5) and a discussion of its use in measurement of the cutaneous pigments (2) have been presented elsewhere. Briefly, the apparatus records the reflectance of the intact living skin, giving an analysis by wave lengths over the entire visible spectrum, from the violet end at 400 mμ to the red end at 700 mμ. In the curve thus obtained, each pigment is identified by its characteristic absorption band. The amount of the pigment is indicated by the degree of absorption at the wave length of the band. Lowering of the reflectance at the particular wave length is therefore proportional to the amount of the pigment present. Previous analysis of normal individuals had demonstrated 5 cutaneous pigments; namely, melanin; a derived material described as melanoid; carotene; reduced hemoglobin and oxyhemoglobin (2). Identification of the pigments in the readings shown in this paper will be facilitated by reference to the spectrophotometric characteristics of each of these substances as illustrated in figure 1.

Observations on Castrate Subjects

In the untreated castrate subjects we were able to demonstrate differences from normal skin involving each of the skin pigments, either as regards its formation, as in the case of melanin, or its distribution, as in the case of hemoglobin and oxyhemoglobin.

In contrast to the normal individual, the skin contained a lesser quantity of blood with an abnormal preponderance of reduced hemoglobin, a lesser amount of melanin and a greater quantity of melanoid and carotene. That these characteristics are attributable to the castrate state is emphasized by the reversion to the normal quantities of pigment when the castrates received testosterone propionate, with subsequent relapse to the castrate condition when the androgenic medication was withdrawn. It will be of interest to follow these changes in some detail, besides calling attention to certain modifications of the relationships noted above.

Hemoglobin and oxyhemoglobin. The most striking and consistent change after treatment with testosterone propionate was the relative increase in the oxyhemoglobin in the cutaneous blood. The resultant rapid rise in the ratio of oxygenated to reduced hemoglobin was accompanied in most areas by an actual increase in the total amount of blood present (fig. 2).

These findings raise a question as to their nature. The increase in oxyhemoglobin might be explained either by a rise in the hemoglobin content of the entire blood stream, or by purely local factors in the cutaneous vascular bed. From our experience with anemic individuals, we can state that it would require a very great increment (approximately 30 to 50%) in the blood hemoglobin to cause spectral variations of the magnitude shown by our subjects. This possibility is ruled out furthermore, by our our inability to find any such substantial change in the red cell count or blood hemoglobin content.

In view of these considerations it follows that the blood vessels of the skin must become dilated after the administration of testosterone. Although the cutaneous vessels are dilated, the blood flow within them might be either decreased or increased. Stagnation of the blood within them would involve a lowering of the local metabolism if the hemoglobin were to remain unreduced. It was found, however, that the general B.M.R. in our patients was not lowered by treatment with testosterone. In some subjects it remained unchanged whereas in others it was slightly increased.

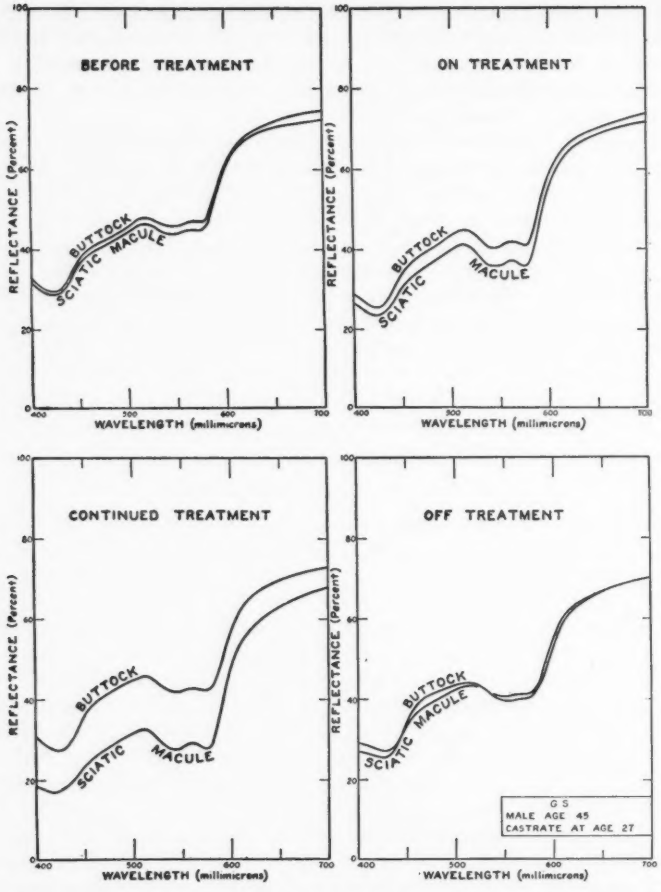

Fig. 2. Curves of the buttock and sciatic macule of castrate G. S. demonstrating that the cutaneous blood flow in the castrate is less than in normal men, that the flow increases upon treatment with androgens, and subsequently decreases when hormone therapy is discontinued.

The designation of macule is given to an area over each ischial tuberosity, which normally possesses a richer blood supply than the remainder of the buttock. In both areas, treatment lowers the curves and sharpens the bands of oxyhemoglobin, denoting an increased cutaneous blood flow. This change is considerably greater in the more vascular macule than in the surrounding area.

Prior to therapy carotene is present in an abnormally large quantity, as shown by the flattening of the curves at 482 mμ. This absorption disappears progessively with treatment and does not return immediately after discontinuance of androgen injections.

We are led, consequently, to the interpretation that in these patients, testosterone propionate produces an increase in the local volume of blood and possibly in cutaneous blood flow.

In normal individuals, the blood content and extent of oxygenation differs greatly in the various cutaneous regions of the body, being most marked in those areas known anatomically to possess large cutaneous capillaries, and where our method has demonstrated large quantities of oxyhemoglobin. In untreated castrates those regional variations in oxyhemoglobin are less pronounced because the entire skin is relatively

poorly vascularized. In androgen treated castrates, however, the increase in oxyhemoglobin was greater in the normally more vascular zones and its increment followed the pattern of relative vascularity which had previously been mapped for the normal subject. Upon cessation of medication these areas again return to a relatively avascular state, this being especially apparent in the more vascular regions (fig. 2).

The rapidity of the vascular action of testosterone propionate was investigated by making observations at 30-minute intervals after a single injection. The curves showed a noticeable effect from one-half to one hour after the injection, the exact

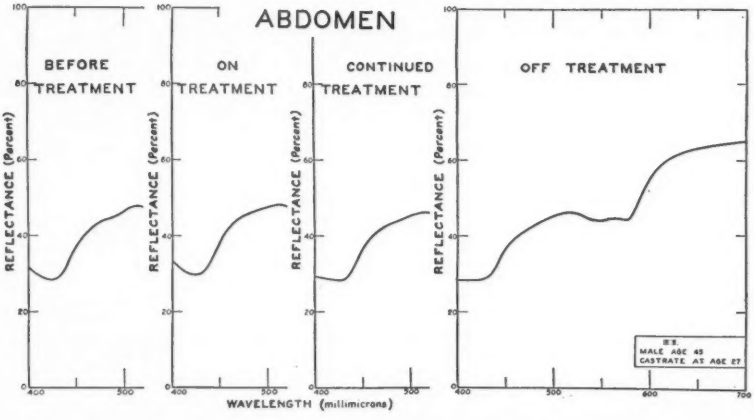

Fig. 3. The diminution of carotene and the increase in melanoid which ensues upon treatment. Curves of the abdomen of castrate, *G. S.*, are arranged to show only a portion of the spectrum. Note the marked absorption band of carotene at 482 mμ in the first curve and its early disappearance upon administration of male hormone substance. Melanoid is indicated by the flattening of the curve at 400 mμ. It increases progressively during treatment and is still strongly evident after treatment has been discontinued for nine weeks.

time varying from subject to subject and in different regions (fig. 4). A peak of effect was usually noticed in from 2 or 3 hours after an injection. There was regression after this maximum, but there remained considerable residual reaction, and upon daily injections, a cumulative effect was noted. Moreover, an injection preceded by a period of daily treatment exerted an influence over a longer period of time than one given at the initiation of treatment.

The low readings for oxyhemoglobin in untreated castrates were associated with an increased quantity of reduced hemoglobin in certain areas. This, we believe, indicates venous stasis. It occurred especially in areas known anatomically to possess large veins, and shown by our method to be rich in reduced hemoglobin, in both normal and castrate men. In such places as a result of androgen administration there was an increase in oxyhemoglobin although the volume of blood diminished (fig. 5).

Diminution in local blood volume with a relative increase in oxyhemoglobin content was also occasionally seen in other, more arterial, zones. In such instances there was an early reduction in the volume of blood which was replaced after continued treatment by an increase in oxyhemoglobin.

The apparent ability of testosterone propionate to increase the vascularity of the skin has led us to use it as a therapeutic agent in a limited number of patients with obliterative vascular disease (6). The results so far have been encouraging.

Carotene. We encountered an increase in the quantity of carotene in the skin of

the entire body of our subjects, and of such magnitude and so consistently as not to be accounted for solely by variation in diet (fig. 2, 4). It was possible to determine that this increased carotene was located primarily in the dermal and subcutaneous fat, where it is normally most abundant. The carotene was apparently not increased in the stratum corneum of the epidermis where it is normally present in small quantity. It will be recalled that the absorption band at 482 mμ falls in a region of high transmission of the overlying skin pigments. Therefore this band is fairly evident whether the carotene causing it be located in the fat or in the epidermis. On the other hand, the band at 455 mμ is obscured by the absorption of both forms of hemoglobin, and

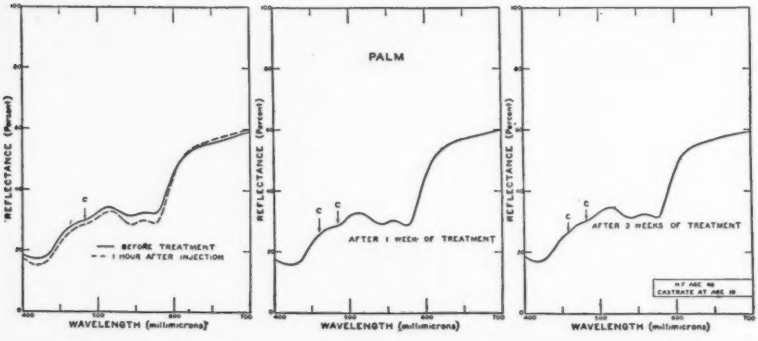

Fig. 4. The temporary increase in epidermal carotene effected by treatment. Curves of the palm of castrate *H. F.*

Carotene, present in large quantity, is increased after 1 week of treatment, and begins to diminish after 3 weeks. Only its absorption (c) at 482 mμ is at first evident, but its second band at 455 mμ is seen after treatment, indicating that carotene has appeared in the stratum corneum, where it is no longer screened by the blood.

Note the rapidity with which testosterone increases the quantity of blood flow shown by the curve made one hour after treatment.

it is not evident unless the carotene is present in the superficial layers of the skin. In the curves of the castrate subjects made before treatment, only the band at 482 mμ is visible, hence, the major part of the carotene is located, as normally, in the fat.

Upon administration of testosterone, the quantity of carotene gradually diminishes until it approximates that seen in normal men. In 2 of the 4 castrates, frequent readings were made during the first few weeks of treatment. In them we noted a temporary increase in carotene in the palms and soles, which after a few weeks showed a diminution along with the remainder of the body (fig. 4). These are regions which under the influence of androgens, receive an unusual quantity of arterial blood, and which, moreover, possess the heaviest stratum corneum of the entire integument. During this period of temporary increase in the carotene content of these localities, one sees both sets of absorption bands of carotene, indicating that a tide of carotene to the stratum corneum has taken place. These findings suggest that the androgenic hormone induces an active mobilization of the large carotene stores of the castrate, accompanied by some actual loss of this material by way of the skin.

Melanin and melanoid. With the exception of our Negro patient, whose normally high melanin content rendered refined estimation of this pigment impossible, the subjects showed melanin values which were unusually low for adult males. Although this deficiency seemed fairly definite, it was clearly not of extreme magnitude. The diminution was noted in both the exposed and unexposed areas of the skin involving, therefore, both primary or native melanin, as well as that which forms secondary to exposure to light. Treatment of these men with testosterone propionate usually in-

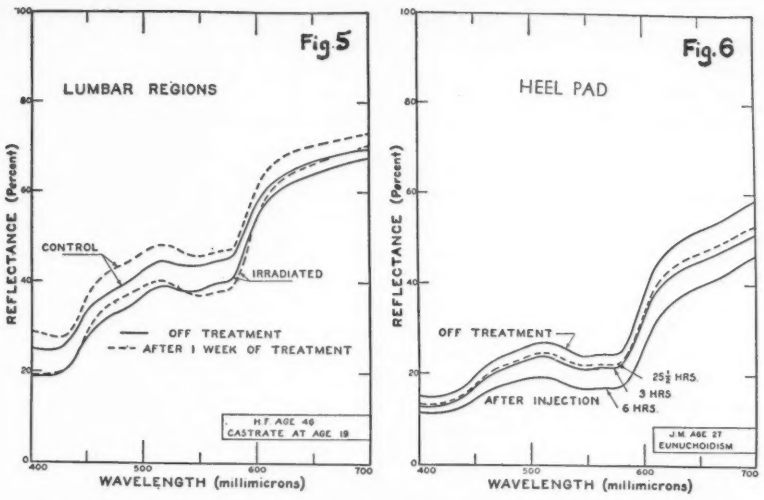

FIG. 5. THE VASCULAR EFFECT OF ANDROGENIC TREATMENT ON A ZONE POSSESSING A LARGE VENOUS BED. The effect in a symmetrical area of skin previously irradiated with ultraviolet light is also shown in these curves of castrate *H. F.*

Upon treatment of the patient with testosterone the amount of hemoglobin was diminished on the control (non-irradiated) side, allowing the curve to rise to a higher level, but with a greater percentage of oxyhemoglobin, since it also sharpens the oxyhemoglobin bands. The flatness of the curve near 400 mμ indicates the presence of considerable melanoid.

The curve of the irradiated region lies at a lower level than that of the non-irradiated side, indicating the presence of more pigment, mainly melanoid. It is problematic whether or not there is more melanin. Reduced hemoglobin is present, however, in great quantity. This is a usual, and long lasting effect of irradiation, previously noted in the normal subject, and presumably due to a loss of venous tone (7). Following injection of *H. F.* with testosterone the percentage of oxygenated hemoglobin is higher; there is a reduction in local blood volume but not to the extent seen in the non-irradiated side.

Fig. 6. THE ACUTE EFFECT OF A SINGLE INJECTION OF A DIFFERENT ANDROGEN IN THE EUNUCHOID PATIENT, *J. M.* (150 mg. OF ANDROSTERONE DISSOLVED IN PEANUT OIL). Hyperemia in this instance was considerable within 3 hours and maximal in 6 hours. It had largely disappeared in 25½ hours.

Note that these curves in a eunuchoid do not show the prominent carotene band so typical in the castrates.

creased the melanin content in all areas, although the response was slow and of small degree. Indeed, in some cases the change was not definite. We were unable to demonstrate any increase upon administration of androgen to the castrate Negro. This may be due, in the main, to the difficulty of distinguishing small variations in melanin when the pigment is present in high concentration.

A more striking abnormality was the presence of an unusual quantity of melanoid. Melanoid is the designation given to a pigment with an absorption band at 400 mμ, demonstrable in the normal person in the sole of the foot, but in other localities only when a great deal of melanin has been formed, as, for example, after irradiation by sunlight (7). We had deduced previously that melanoid is a degradation product of melanin, but in our present subjects it appears to be formed independently of that material. Our patients had avoided all exposure of their bodies (except the head and hands) to sunlight, for many months prior to the present observations. Nevertheless, they showed large quantities of melanoid over the entire skin and face, to a degree resembling that seen during the recession of tanning (fig. 3). As in the case of melanin, we failed to demonstrate any variation in the melanoid content of our Negro patient. While the administration of testosterone propionate to our castrates increased the melanin content but slightly, it stimulated the production of excessive quantities of melanoid (fig. 3). This suggests that testosterone propionate, although it corrects

many of the results of castration, does not reproduce all the effects of the normal testicular hormones. The apparent failure may, however, depend on the effects of castration on other endocrine glands, such as the adrenal or pituitary, which are concerned with the formation of melanin. Moreover, changes in oxygenation of the cutaneous blood, such as we have found, may affect melanin chemically, and lead to its conversion into the related compound, melanoid (8). On the other hand, it is possible that the correction of certain of the castrate phenomena by testosterone propionate may require more time than we have allowed in our experiments. However, this alternative is not supported by our observations on *H. M.*, who after more than a year of practically daily injections of a minimum of 20 mg. of testosterone propionate, still exhibited a greater quantity of melanoid than occurs in normal men.

The effect of ultraviolet irradiation was studied in the 3 white castrates. In all, the exposed areas were noticeably darker than the surrounding skin within 2 or more weeks, at a time when tanning would normally be pronounced. Examination, however, disclosed but little melanin (fig. 5). Indeed, in one patient (*H. F.*), there was no measureable increase in this substance. On the other hand, melanoid was increased in every instance. Reduced hemoglobin was the pigment which was most strikingly increased, and was probably most important in the darkening of the exposed areas. In a previous study of the normal we had observed a similar but less pronounced post-irradiation increase in hemoglobin, and interpreted it as being due to a long lasting venous stagnation (7).

Treatment with testosterone propionate promptly increased the contrast between the irradiated and non-irradiated zones (fig. 5). In the irradiated areas an increase in melanin was barely noticeable in one patient (*G. S.*) and not at all in the other 2. Melanoid, on the other hand, was definitely increased in all 3 subjects. A substantial diminution in the content of reduced hemoglobin, and an increase in the relative proportion of oxyhemoglobin were also observed, although this reaction was more pronounced in the non-irradiated areas than in the exposed zones.

Observations on Eunuchoid Subjects

The results obtained in this group varied with the eunuchoid state of each subject. *H. M.* who possesses one poorly-functioning cryptorchid testis (only 13.5 I.U. of urinary androgens per 24 hours), the other, normal testis having been removed, showed the general bodily and functional characteristics of a total castrate, as well as spectrophotometric findings identical with those described above in the true castrates. He is, for practical purposes, a castrate and differs from the remaining 4 eunuchoid individuals which are described below.

Three of the remaining four patients were eunuchoids apparently with secondary hypogonadism, since the titers of urinary gonadotropic substance were not elevated as in castrates and other cases of primary hypogonadism, but were instead below normal. These 3 and the dwarf, not only showed no complete resemblance to the castrates, but also differed considerably from each other. Particularly striking was the apparent adequacy of the cutaneous circulation, both as regards the volume of blood and the ratio of reduced to oxyhemoglobin. Nevertheless, upon administering androgenic hormones the skin showed an increased volume of blood with a higher percentage of oxyhemoglobin, but no evidence of diminution in blood volume in the more venous regions of the body (fig. 6).

These individuals varied markedly in their ability to form melanin, and there appeared to be no correlation between the quantity of melanin in the skin and the amounts of gonadal hormones excreted in the urine. Thus *M. D.*, with a daily output of 24 I.U. of androgens and 15 I.U. of estrogens, was strikingly deficient in cutaneous melanin; while *E. M.*, with a value of 22 I.U. of androgenic and 22 I.U. of estrogenic

substances, was extraordinarily melanotic, with a skin color which would match that of many mulattoes. *J. M.*, brother of the latter patient, excreting 9 I.U. of androgens and 31 I.U. of estrogens daily, and the dwarf, *R. S.* were only moderately pigmented. *E. M.* and *J. M.*, both of Semitic ancestry, came from a family whose complexion was very dark.

Melanoid was detectable in quantities slightly greater than normal, both before and after treatment in *E. M.*, *J. M.* and *R.S.* Melanoid substance was not pronounced in *M.D.*, the patient whose melanin content was extremely low.

Finally, none of this group showed the increased skin carotene so striking in the castrates.

DISCUSSION

Although it is generally true that the gonads may influence the pigmentation of the skin it is difficult to compare directly the findings in various species of animals. Our observations indicate that in man the testes affect the entire integument, the degree of influence in each locality depending on various factors, including the relative vascularity and the rate of melanogenesis of the region. In other animals, however, the available observations indicate that the gonads act principally on certain specialized areas of the skin or its appendages.

Known examples in these animals of the influence of the testes on the skin and its appendages are pertinent to the present discussion. The cock's comb resembles the human skin from the standpoint of changes in its blood vessels. Thus, castration causes a shrinkage in size of the arteries, arterioles and capillaries of the comb with a flattening of the ordinary high dermal papillae. Moreover, here, as in man, an enlargement of the veins accompanies the diminution of the arterial caliber. Similarly, the comb of the castrated cock reverts to normal after the administration of testicular extract (9, 10).

When we examine the possible influence of the testes on melanin formation, we are confronted by the genetic aspects of the secondary sex characters. It has been clearly shown that sexual dimorphism is dependent more on the particular chromosomal pattern which at the outset established the sex of the individual, than on the activity of the gonads. Thus the plumage of many birds retains its sexually dimorphic coloration after castration. In such instances it is obvious that the activity of the gonads is not necessary for the maintenance of the pigment formation, including that of melanin. Gonadal regulation of plumage color must be regarded as being subsidiary to the more fundamental factors of genetic control (11). By way of example we may cite the observations of Keck (12), and of Witschi and Woods (13), that while castration of the male English sparrow (*Passer domesticus*) leaves unaltered the color of the plumage, it does inhibit the formation of melanin in the bill. A yellow pigment appears under these conditions, but whether or not this material is melanoid we cannot say.

Data on carotene changes in the skin of animals are lacking, so that comparison with our findings in man is not possible.

SUMMARY

The cutaneous vascularity and pigmentation of 4 human male castrates (including a Negro), and 5 eunuchoid patients were studied before, during and after treatment with testosterone propionate. Determinations were made with the aid of the Hardy recording spectrophotometer. The castrates were found to possess a generally diminished quantity of hemoglobin in the skin, and of that present, a higher proportion than in normal men is in a reduced state. This is interpreted as an indication that the cutaneous vascular bed of castrates is smaller and has less blood flowing through

it than in normal individuals. Conversely, in those cutaneous areas characterized by a large venous bed, there is a greater amount of reduced hemoglobin than in normal men, evidence that the veins are in a dilated condition. These changes were reversed by administration of testosterone propionate: in areas containing a large amount of venous blood, the volume of blood and the proportion of reduced hemoglobin diminished. In more arterial regions of the integument, on the contrary, the volume of blood was increased and it likewise contained more oxyhemoglobin. The action of male hormone began within one hour after injection, reaching a maximum effect in about 2 to 3 hours.

Carotene was present in the skin of untreated castrates in excessive quantity. Administration of testosterone propionate brought this pigment to the level found in normal men; there was a temporary increase of the pigment in the stratum corneum of the palms and soles, indicating that some carotene was lost in disquamated cells.

The content of melanin in the skin of untreated castrates is slightly subnormal, but it is accompanied by a large amount of the related substance, melanoid. Treatment with androgens resulted in an increase in both of these pigments, but especially in the latter.

Ultraviolet irradiation of the untreated castrate's skin did not consistently stimulate the production of melanin, but did induce the formation of a large quantity of melanoid. The limited degree of tanning of the irradiated area was attributable to the excess of melanoid, as well as to the accentuation of the usual blood stagnation which follows irradiation. Treatment with testosterone propionate rendered the irradiated areas even more prominent, mainly by further increasing the melanoid, as well as by the more rapid reversion of the non-irradiated skin to its normal vascular state.

The skin of one eunuchoid patient with marked insufficiency of testicular junction resembled the castrates quite completely. The skin of 3 other eunuchoids with secondary hypogonadism was very nearly normal, showing no carotene change, and a variable modification of the other pigments.

It is concluded that androgens have a profound influence on the vascularity and pigmentation of the entire human skin. Comparison is made with the known similar effects of the gonads in other animals in whom, however, the gonads have been noted to affect principally localized zones of skin or cutaneous appendages. It is to be expected that further study might bring out more evidence of the widespread effect of the sex hormones, beyond the confines of the accepted secondary sex characters of the individual.

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THE ACTION OF VARIOUS STEROID HORMONES ON THE TESTIS

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EVER SINCE Moore and Price (1) formulated the hypothesis that the hormones of the sex glands cause atrophy of the gonads because they inhibit the gonadotropic hormone secretion of the pituitary, numerous investigators published experiments showing that androgens and estrogens produce atrophy of the gonads in both sexes. Since gonadal atrophy is one of the most readily developing consequences of non-specific damage (2), the first observations in which impure and often toxic organ or urine extracts were used, are inconclusive. However, it has now definitely been proven that even pure crystalline sex hormones are capable of producing gonad atrophy. As regards the relevant literature dealing with the action of such substances on the mammalian testis, the subject under consideration in this paper, it will be recalled that testis atrophy had been produced with crystalline estrogens in the rat (3–6), the rabbit (7) and several other species. Progesterone (8) and crystalline androgens such as testosterone (9) proved to have the same effect as shown by experiments in the rat. It was of particular interest that desoxycorticosterone acetate which is not a gonadal hormone, likewise produces testis atrophy in the rat and mouse especially if administered before puberty (8, 10). However, the evidence on this subject is contradictory since other investigators claimed to have observed stimulation of the testis in animals treated with these hormones. Thus adrenal cortical extracts accelerate spermatogenesis in the immature rat according to Corey and Britton (11) and Klein (12), and similar observations have been reported following treatment with estrogens in the cat (13) and rat (14). After the injection of various crystalline androgens, an increase in testis size (6, 15) and proliferation of the seminiferous epithelium (16) have likewise been noted in immature rats.

The gonadotropic action of steroids, especially androgens, is of considerable clinical significance since these are commonly in use for the treatment of hypogonadism in man. But while it is generally agreed that androgen treatment stimulates the accessory sex organs of eunuchoid patients and some clinicians even reported descent and enlargement of cryptorchid, underdeveloped testes (17–19), others warned that "though male sex hormone will produce an enlargement of the penis and scrotum and growth of pubic hair, it has a marked tendency to cause a shrinkage in the size of the testicle, both descended and undescended" (20). More recently McCullagh and McGurl (21) concluded from observations made in a series of hypogonadal adults that testosterone propionate causes a pronounced, though transitory, fall in the total sperm production, a finding which would appear to cast some doubt on the validity of the suggestion of Lower et al. (22) to use androgens in the treatment of male sterility. In an effort to elucidate this problem, Zuckerman (23) performed experiments in immature monkeys which he treated with testosterone propionate, but he failed to show any gonadotropic action in these primates.

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In this communication we wish to report on a series of systematic investigations concerning the gonadotropic action of pure steroids which indicate that the contradictory findings of previous workers are, for the most part, probably due to differences in the dosage in which their preparations were administered. This appears likely since we found that, at least in the case of testosterone, relatively small doses cause testis atrophy while larger doses have no such effect and may actually prevent the involution of the testes which is otherwise produced by various agents.

EXPERIMENTS

Testosterone

Experiment 1. Two groups of 12 adult mice of the dba strain (Little's dilute brown) were selected. In the first group, 6 animals received 19 daily injections of 5 mg. of testosterone dissolved in 0.2 cc. of peanut oil, while the remaining 6 served as controls and were given a similar quantity of cholesterol (a hormonally inactive sterol) in oil. The second group was treated in the same way for 40 days. Table 1 summarizes the data. In this as in all our tables, the average testis and body weight is given in grams and the range of the individual testis weights is indicated in brackets.

TABLE 1. EFFECT OF TESTOSTERONE ON TESTIS OF ADULT MOUSE

Treatment	No. of mice	Body weight	Testis weight	Histology
Cholesterol 5 mg.—19 days	6	21	0.158 (142–183)	Normal
Testosterone 5 mg.—19 days	6	21	0.148 (125–187)	Leydig cell atrophy
Cholesterol 5 mg.—40 days	6	23	0.154 (130–193)	Normal
Testosterone 5 mg.—40 days	6	23	0.130 (120–147)	Leydig cell atrophy

It appears that in the mature mouse, a large dose of testosterone produces no significant decrease in testis weight, and histologically only interstitial cell atrophy without damage to the tubular epithelium.

Experiment 2. Six adult albino rats received 2 mg. of testosterone in 0.2 cc. of peanut oil daily subcutaneously, 6 received 10 mg. of the hormone daily in 0.4 cc. of oil, 6 were treated with a similar quantity of cholesterol in oil and 6 served as untreated controls. Table 2 summarizes our results.

TABLE 2. EFFECT OF TESTOSTERONE ON TESTIS OF ADULT RAT

Treatment	No. of rats	Body weight	Testis weight	Histology
Untreated	6	203	2.52 (2.31–2.96)	Normal
Testosterone 2 mg.—20 days	6	194	1.95 (1.70–2.25)	Moderate tubular atrophy with some giant cell formation—Leydig cell atrophy
Cholesterol 10 mg.—20 days	6	216	2.64 (2.39–2.91)	Normal
Testosterone 10 mg.—20 days	6	170	2.42 (2.17–2.63)	Leydig cell atrophy only

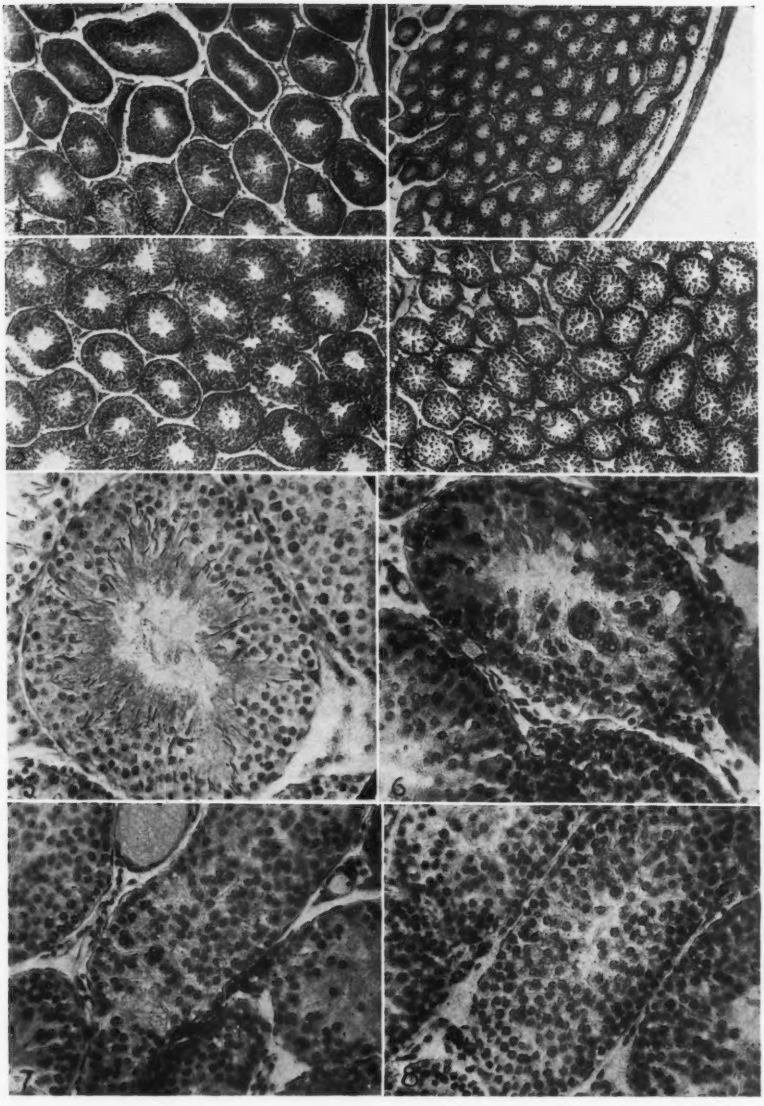

Fig. 1. Low magnification of testis of a 40-day-old normal rat. Fig. 2. Hypoplastic testis of a 40-day-old rat chronically treated with desoxycorticosterone acetate (same magnification as fig. 1). Fig. 3. Low magnification of testis of a 70-day-old normal rat (same magnification as fig. 1). Fig. 4. Hypoplastic testis of a 70-day-old rat treated with desoxycorticosterone acetate until 40th day of life then given 30-day rest period (same magnification as fig. 1). Fig. 5. High magnification of testis in normal adult rat. Fig. 6. Testis atrophy in desoxycorticosterone acetate treated adult rat. Note Leydig cell atrophy. The tubules show fully developed giant cells and the manner in which secondary spermatocytes aggregate to form such giant cells (same magnification as fig. 5). Fig. 7. Early stage of giant cell formation in desoxycorticosterone acetate treated adult rat. Note typical early clumping of secondary spermatocytes with loss of cell boundaries (same magnification as fig. 5). Fig. 8. Earliest recognizable stage of tubular atrophy in desoxycorticosterone acetate treated adult rat. The secondary spermatocytes have undergone some cloudy swelling, the nuclei are enlarged and pale while sperm formation is at a minimum (same magnification as fig. 5).

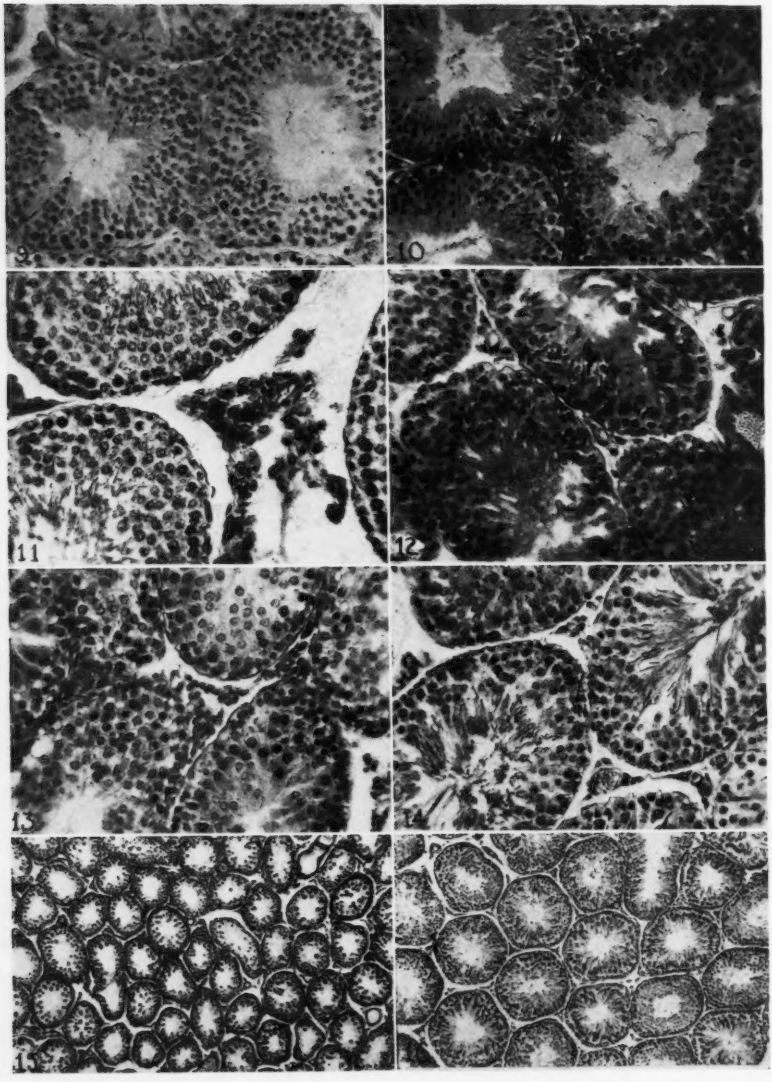

Fig. 9. Normal Leydig cells in testis of adult mouse (high magnification). Fig. 10. Leydig cell atrophy in adult mouse treated with progesterone (same magnification as fig. 9.) Fig. 11. Normal Leydig cells in testis of adult rat (high magnification). Fig. 12. Leydig cell atrophy in testis of progesterone treated adult rat. (same magnification as fig. 11). Fig. 13. Leydig cell atrophy in testis of estradiol treated adult rat (same magnification as fig. 11). Fig. 14. Leydig cell atrophy in testosterone treated adult rat (same magnification as fig. 11). Fig. 15. Inhibition of spermatogenesis in testis of estradiol treated young rat (same magnification as fig. 16). Fig. 16. Apparently normal testis tubules in young rat treated with testosterone and the same dose of estradiol as was given to the animals shown in figure 15. Note the neutralization of the estrogen effect by the androgen (low magnification).

These data revealed the unexpected fact that although testosterone invariably causes Leydig cell atrophy, it damages the tubular tissue only when relatively low doses are administered.

Desoxycorticosterone Acetate

Experiment 1. Beginning on the day of birth, 5 of a litter of 7 male hooded rats received 30 daily subcutaneous injections of 1 mg. of desoxycorticosterone acetate in 0.1 cc. of peanut oil, followed by 10 daily injections of 2 mg. in 0.2 cc. of oil. Two of the treated animals were killed immediately after the last injection while 3 were killed only after having been maintained for 30 days without treatment.

The 2 animals killed on the fortieth day of treatment, having received a total of 50 mg. of the hormone, showed testis weights of 63 mg. and 109 mg. as against 1.725 gm. in an untreated litter mate killed on the same day. The 3 animals in which treatment was suspended during the next 30 days showed at the end of that time, an

TABLE 3. EFFECT OF DESOXYCORTICOSTERONE ACETATE ON THE TESTIS OF THE ADULT RAT

Treatment	No. of rats	Body weight	Testis weight	Histology
Untreated	6	203	2.527 (2.310–2.960)	Normal testis
Desoxycorticosterone acetate 2 mg.—20 days	6	194	2.327 (2.115–2.585)	No histological change
Desoxycorticosterone acetate 10 mg.—20 days	6	159	1.440 (0.680–2.135)	Marked tubular and Leydig cell atrophy with giant cells

average testis weight of 1.278 gm. (1.350, 2.092, 0.392) as against 1.430 gm. in an untreated litter mate killed that day. In all the hormone treated animals, the interstitial tissue of the testis was markedly atrophic while the tubules showed pronounced inhibition of spermatogenic activity (fig. 1–4).

We conclude that this small experimental series supports the view that desoxycorticosterone acetate inhibits the development of the immature rat testis, produces interstitial atrophy and inhibits normal spermatogenesis. This effect is still operative for a considerable time after cessation of the injections.

Experiment 2. Eighteen adult male albino rats were used. Six animals received 2 mg. and 6 10 mg. of desoxycorticosterone acetate subcutaneously in 0.4 cc. of peanut oil daily subcutaneously while the remaining 6 were untreated. At the end of the period of injection, the testes were weighed and histologically examined. Table 3 summarizes the data.

We see that in the adult rat prolonged treatment with relatively small doses of desoxycorticosterone acetate elicits no change in the normal testis structure and is accompanied by no significant change in testis weight. On the other hand, prolonged treatment with a high dose of desoxycorticosterone acetate produces a significantly smaller testis and the histological picture of marked interstitial and tubular atrophy accompanied by the formation of giant cells from secondary spermatocytes (fig. 5–8).

Progesterone

Experiment 1. Male adult black mice of an average body weight of 25 gm. were selected and divided into 2 groups. In the first group, 6 animals received 20 daily subcutaneous injections of 3 mg. of progesterone while a similar group served as un-

treated controls. In the second group, 6 animals received the same daily dose of the hormone for 40 days and 6 served as untreated controls.

In the group treated for 20 days, the average testis weight was 136 mg. as against 161 mg. for the normals. Similarly the group treated for 40 days showed an average testis weight of 126 mg. as against 169 mg. for their controls. Histologically the interstitial tissue was markedly atrophic in both treated groups while the tubular epithelium was entirely normal (fig. 9, 10).

From these data, we conclude that in the mouse, progesterone given in this dosage produces a moderate decrease in testis weight but that its effects are confined to the interstitial tissue—the seminferous epithelium remaining entirely unharmed.

Experiment 2. Twelve young mature male albino rats having an average body weight of 135 gm. were divided into 2 groups. Six received 12 daily subcutaneous

TABLE 4. EFFECT OF PROGESTERONE ON THE TESTIS OF THE ADULT RAT

Treatment	No. of rats	Body weight	Testis weight	Histology
Untreated	6	216	2.773 (2.533–2.983)	Normal testis
Progesterone 2 mg.—20 days	6	206	2.420 (2.241–2.621)	Slight Leydig cell atrophy—normal tubules
Progesterone 10 mg.—20 days	6	190	2.057 (1.767–2.317)	Leydig cell atrophy—normal tubules

injections of 4 mg. of progesterone in 0.2 cc. of peanut oil, while the remaining 6 served as untreated controls. At the end of the injection period, the animals were killed and the testes taken for weight and histological study.

In the treated group, the testes weighed 2.07 gm. (1.68, 2.55) as against 2.2 gm. (1.67, 2.4) in the untreated controls. The Leydig cells were atrophic in the treated group but the seminiferous epithelium showed no obvious change.

From these data, we conclude that progesterone in this dosage is relatively ineffective on the male rat gonad and produces only slight interstitial cell atrophy.

Experiment 3. Eighteen adult male albino rats were used. Six animals received daily 2 mg., and 6 10 mg. doses of progesterone in peanut oil subcutaneously while the remaining 6 acted as untreated controls. At the end of the injection period the testes were weighed and histologically examined. Table 4 summarizes the data.

It appears that in the adult rat, progesterone in low doses is unable to produce any change either in the weight or histological appearance of the testis and even relatively high doses elicit only a moderate atrophy (fig. 11, 12).

Since it has definitely been established (24) that in the female, small doses of progesterone decrease the size of the ovary while large doses increase it as a result of luteinization, it appeared of interest to establish whether or not the lack of more pronounced testis atrophy in these experiments might not be due to the fact that progesterone exerts a marked direct stimulating effect on the testis. Such a direct effect may most clearly be demonstrated in hypophysectomized animals. That androgens inhibit the testicular atrophy which usually follows after hypophysectomy has clearly been shown by numerous experiments (25–28). We may confirm these observations but since this effect received adequate attention in the literature, it appears unnecessary to report our relevant findings in detail. The action of progesterone in the hypophysectomized male, on the other hand, received but little attention although Nelson (26, 29, 30) made repeated cursory reference to the fact that this compound may also prevent testis involution after ablation of the pituitary. We know

from a letter which Professor Nelson wrote to one of us (H.S.) that he will soon publish the conditions under which his experiments were performed, but since no such report is available at this time, it would perhaps be useful to communicate one of our relevant experiments which fully confirms his observations.

Progesterone after Hypophysectomy

Twenty young male albino rats having an average body weight of 88 gm. were used. In the first group, 5 animals received 9 daily injections of 2 mg. of progesterone in 0.1 cc. of peanut oil followed by 8 daily injections of 10 mg. of the hormone in 0.2 cc. of oil, while the same number of control animals received a similar quantity of peanut oil only. In the second group, all animals were hypophysectomized and immediately after the operation, 5 of them received progesterone and 5 oil in the same manner as the rats of the first group. Table 5 summarizes the data.

TABLE 5. EFFECT OF PROGESTERONE ON THE TESTIS OF THE HYPOPHYSECTOMIZED RAT

Treatment	No. of rats	Testis weight	Histology	
			Interstitial cells	Tubules
Peanut oil	5	1.179 (0.593–1.735)	Normal	Normal
Progesterone	5	1.374 (1.227–1.566)	Slightly atrophic	Normal
Hypophysectomy and peanut oil	5	0.452 (0.169–0.590)	Atrophic	Arrest in spermatogensis with some giant cell formation
Hypophysectomy and progesterone	5	0.916 (0.603–1.25)	Atrophic	Normal full range of spermatogenesis

From these data it is apparent that progesterone in the high dosage used produces no significant change either in the gross weight or structure of the intact young rat testis. However, in the hypophysectomized animal, this same dosage exerts an apparently direct action on the testis, maintaining it at almost normal size. This action is due only to the maintenance of the seminiferous epithelium while the atrophy of the Leydig cells remains unmodified.

Combined Treatment with Various Steroid Hormones

Adult male albino rats having an average body weight of 173 gm. were injected subcutaneously during 20 days with the various steroids, the daily dose of which was dissolved in 0.2 cc. of peanut oil in each case. Since non-specific damaging agents also cause gonadal atrophy it appeared advisable to compare the actions of the steroids with those of a toxic agent. For this purpose bi-daily subcutaneous injections of 0.2 cc. of a 4% aqueous formaldehyde solution were given alone and in combination with steroid hormones. Table 6 summarizes our findings.

It appears that in the dosage used, testosterone, estradiol, progesterone and formaldehyde are each capable of producing interstitial cell atrophy, while in addition to this, estradiol and testosterone produced a significant loss in the gross weight of the gonad and marked changes in the seminiferous epithelium. In the case of combined treatment with testosterone and desoxycorticosterone acetate or testosterone and formaldehyde; and in the group receiving desoxycorticosterone acetate with estradiol, the atrophy producing effect of the drugs is summated, both with respect

TABLE 6. EFFECT OF COMBINED TREATMENT WITH VARIOUS STEROID HORMONES ON TESTIS OF ADULT RAT

Treatment	Testis weight	Histology	
		Interstitial cells	Tubular epithelium
Untreated	2.527 (2.310–2.960)	Normal	Normal
Desoxycorticosterone acetate—2 mg.[1]	2.327 (2.115–2.585)	Normal	Normal
Testosterone—2 mg.[1]	1.959 (1.70 –2.251)	Atrophic	Moderate atrophy with some giant cells.
Estradiol—300	0.645 (0.508–0.891)	Atrophic	Marked arrest in spermatogenesis with some giant cells
Progesterone—2 mg.[1]	2.420 (2.241–2.621)	Slightly atrophic	
Formaldehyde—0.2 cc. twice daily	2.308 (2.107–2.618)	Moderately atrophic	Moderately atrophic
Desoxycorticosterone acetate—2 mg. Testosterone—2 mg.	1.811 (1.431–2.167)	Atrophic	Slight atrophy
Desoxycorticosterone acetate—2 mg. Estradiol—300	0.531 (0.298–0.726)	Atrophic	Arrest in spermatogenesis with giant cells
Estradiol—300 Progesterone—2 mg.	1.263 (0.905–1.759)	Atrophic	Arrest in spermatogenesis with giant cells
Testosterone—2 mg. Estradiol—300	2.113 (1.941–2.248)	Atrophic	Normal
Testosterone—2 mg. Progesterone—2 mg.	2.313 (2.033–2.448)	Atrophic	Normal
Testosterone—2 mg. Formaldehyde—0.2 cc. as above	1.907 (1.531–2.201)	Moderately atrophic	Moderately atrophic
Testosterone—2 mg. Progesterone—2 mg. Estradiol—300	1.972 (1.392–2.372)	Atrophic	Normal

[1] These groups are identical with those summarized in previous tables and are given here merely for comparative purposes.

to testis weight and as regards the histological signs of involution in the spermatogenic epithelium and the Leydig cells. On the other hand, combined administration of estradiol with progesterone, testosterone or a combination of the two latter compounds, decreases the involution of the seminiferous epithelium and consequently inhibits the loss in testis weight. This we ascribe to the direct testis stimulating action of progesterone and testosterone. In the small dose used in this experiment testosterone was unable to inhibit the testis involution caused by formaldehyde but as we shall see later, larger doses of the same compound are very active in this respect. We wish to place special emphasis on the fact that the interstitial cells undergo atrophy with all dosage levels of all the steroid hormones described in this communication (fig. 11–14). We interpret this as an indication that unlike the seminiferous epithelium the Leydig cells are not stimulated by the steroids. This view receives further support from our observations indicating that in the hypophysectomized rat neither androgens nor progesterone can maintain the interstitial cells even though they prevent or delay the involution of the seminiferous epithelium.

These findings lead us to the conclusion that the apparently contradictory observations regarding the action of testosterone on the testis, mentioned in the introductory review of the literature, are best explained by the assumption that this hormone acts on the testis by way of two distinct and antagonistic mechanisms. These are the depression of the gonadotropic hormone production by the hypophysis, which is an indirect inhibitory effect, and the stimulation of the seminiferous epithelium itself, which may be regarded as a direct trophic action. The threshold dose for the pituitary inhibiting effect is apparently lower than that for the direct gonadotropic action.

All the experiments reported in table 6 as well as those described earlier in this paper and elsewhere in the literature appear to be in good accord with this hypothesis

TABLE 7. EFFECT OF TESTOSTERONE AT VARIOUS DOSAGE LEVELS ALONE AND IN COMBINATION WITH OTHER COMPOUNDS ON TESTIS OF YOUNG RAT

Treatment	Testis weight	Histology	
		Interstitial cells	Tubules
Untreated	2.063 (1.750–2.273)	Normal	Normal
Testosterone—1 mg.	1.580 (0.958–1.947)	Atrophic	Normal appearing but of subnormal size
Testosterone—10 mg.	2.051 (1.831–2.157)	Atrophic	Normal
Estradiol—300	0.354 (0.259–0.452)	Atrophic	Arrest in spermatogenesis with giant cells
Formaldehyde (0.2 cc. b.i.d.)	1.547 (1.257–1.819)	Atrophic	Normal appearing but of subnormal size
Testosterone—10 mg. Estradiol—300	1.726 (1.135–2.263)	Atrophic	Normal appearing but slightly less than normal size
Testosterone—10 mg. Formaldehyde (0.3 cc. b.i.d.)	1.927 (1.200–2.489)	Atrophic	Normal appearing but slightly less than normal size

which also helps in understanding the otherwise puzzling fact that two hormones given in a dosage in which either of them would cause gonad involution have a less pronounced effect if given simultaneously. However, to prove this theory conclusively it appeared important to establish beyond doubt that a steroid, e.g. testosterone, has different effects on the testis depending on the dosage in which it is used. In order to show this, we performed the following experiment on young male albino rats having an average body weight of 77 gm. Young animals were used because we knew from previous experience that it is easier to produce inhibition of testis development than atrophy of a fully mature gonad. Forty-two such rats were divided into 7 groups of 6 animals each. They were treated with various steroids, the daily dose of which was dissolved in peanut oil and administered subcutaneously. After 20 days of treatment, the animals were killed and the testes weighed and histologically examined. Table 7 summarizes the details of this experiment.

From these data, it is apparent that a low dose of testosterone inhibits testis development, while a high dose exerts no such effect. In fact such high doses are able to maintain the testis weight at a normal level in spite of simultaneous treatment with estrin or formaldehyde in doses which by themselves cause gonad involution (fig. 15, 16).

As incidental observations we noted in the above experiment that estrin alone enlarged the pituitary to 11 mg. as against a normal of 5 mg., while in the combination of estrin with 10 mg. of testosterone the pituitary remained normal in size (5 mg.). Furthermore the adrenals were slightly smaller than normal in the group treated with 1 mg. of testosterone daily and larger than normal in the group treated with 10 mg. daily.

DISCUSSION

The above experiments clearly indicate that small doses of androgens cause testis atrophy while large doses have no such effect and actually counteract involution of the testes in various experimental conditions which normally lead to testis atrophy. This finding helps to understand why previous investigators of this problem, who did not consider the importance of the dosage factor, obtained contradictory results. At first sight, it appears difficult, however, to understand the reason for these differences in action. The fact that androgens and progesterone stimulate the testis of hypophysectomized rats indicates beyond doubt that these hormones exert a direct gonadotropic action in males which is not mediated by the pituitary. We feel that all the known relevant facts are best in accordance with the assumption that in addition to this direct stimulating action, the steroids also have the property of inhibiting the gonadotropic hormone secretion of the pituitary. It appears that at low dosage levels this latter action prevails inasmuch as small doses of various steroids, particularly the androgens, decrease gonadotropic hormone secretion much more than can be compensated for by their direct testis stimulating effect. On the other hand, very large doses have a sufficiently pronounced direct stimulating action to compensate for the loss of pituitary gonadotropic hormone production. From this it would appear that if the hormonal mechanisms regulating testis function are the same in man as they are in the rat and mouse, there would be less danger of damaging the testes of hypogonadal patients with large doses of androgens than with carefully administered small doses. In fact, it appears very probable that the large doses would stimulate gonadal development, or at least inhibit gonadal involution just as in the rats of our present series, massive doses of testosterone inhibited the involution of the testes which is normally caused by estrogens or non-specific damage due to injection of toxic doses of formaldehyde.

Our experiments indicate furthermore that only the seminiferous epithelium is stimulated by large doses of androgens, while the Leydig cells undergo atrophy irrespective of the dosage used. If we accept the prevailing view according to which these cells are the main source of androgen formation, this observation would be in conformity with the law of 'compensatory atrophy' (32) according to which overdosage with a certain hormone causes involution of the cells normally responsible for for its elaboration. The fact that other steroid hormones, namely estrogens, desoxycorticosterone and progesterone, likewise result in Leydig cell atrophy on the other hand is an excellent example of the so-called transferred compensatory atrophy (32). This is an involution of endocrine cells due to overdosage with a hormone other than that normally produced by them, though usually closely related to it.

That unlike the androgens, the estrogens and desoxycorticosterone acetate failed to stimulate the gonads will require further study in hypophysectomized animals in which the stimulating action of steroids are most readily detectable, since they are not counteracted by the simultaneous inhibition of hypophyseal gonadotropic hormone production. It is possible that estrogens and desoxycorticosterone acetate exhibit mainly a gonad inhibitory action because they are very potent inhibitors of pituitary gonadotropic hormone production, while their testis stimulating action, if any, is

comparatively slight. In the case of progesterone, however, such a stimulating effect is clearly noticeable since this steroid not only counteracts the gonadal atrophy of hypophysectomized rats but also inhibits the testis atrophy normally produced by testosterone and estradiol (table 6).

SUMMARY

Experiments in the rat and mouse indicate that small doses of testosterone propionate or testosterone cause a pronounced decrease in testis weight while large doses have no such effect.

Androgens and progesterone, especially when administered in large doses counteract the testis atrophy producing effect of estrogens, non-specific damaging agents such as formaldehyde etc. Small doses on the other hand, actually aggravate the atrophy produced by such means. In hypophysectomized rats, progesterone as well as androgens inhibit involution of the seminiferous epithelium but not of the Leydig cells. From this it is concluded that if the hormonal control of the human testis obeys the same laws that govern the development of the testis in rodents, there would be less danger of causing testis atrophy in eunuchoid patients if they were treated with large doses of androgens than if small doses were administered. In fact large doses may prove to stimulate not only the accessory genitalia but even the male gonad itself.

We assume that various steroids, especially the androgens and progesterone, exert a direct stimulating action on the seminiferous epithelium particularly when they are administered in large doses. In small doses, on the other hand, this action is not detectable since it is overcompensated by the testis atrophy which results from the ability of these steroids to inhibit the gonadotropic hormone secretion of the pituitary. Desoxycorticosterone acetate and estrogens also inhibit gonadotropic hormone production and consequently cause testis atrophy, but so far they have not been shown to exert any direct testis stimulating effect.

The Leydig cells undergo atrophy following treatment with androgens, estrogens, progesterone or desoxycorticostrone acetate alone and in combination at any dosage level tested. In the case of the androgens this is to be regarded as a 'compensatory atrophy' while in the case of the other steroids it probably represents an example of the so-called transferred compensatory atrophy.

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THE FROG TEST (*XENOPUS LAEVIS*), AS A RAPID DIAGNOSTIC TEST FOR EARLY PREGNANCY[1]

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In 1930, Hogben (1) published his report on the use of the South African clawed toad, *Xenopus laevis* Daud, showing that extracts of the anterior lobe of the pituitary caused spontaneous ovulation in the mature female Xenopus. Following this original observation Shapiro and Zwarenstein (2) and Bellerby (3) working independently, simultaneously suggested the use of the Xenopus as an excellent animal for testing for pregnancy.

Since the first reports of these early investigators many other substantiating results have been added to the literature (4, 5, 6). These observers used extracts of urine of pregnancy, injected the animals with concentrates of the anterior pituitary-like hormone (APL) derived from the urines and within 6 to 18 hours could establish the diagnosis of pregnancy. From a series of approximately 600 tests, an accuracy in diagnosis when checked with the Aschheim-Zondek test of 99% was obtained. Crew has become so enthusiastic with his results that in his pregnancy diagnosis laboratories in Edinburgh, he prefers the Xenopus test to the Friedman test for pregnancy.

The technic employed by these workers consisted of a simple alcohol or acetone extraction of the suspected urine with the subcutaneous injection of a 1 cc. extract (equivalent to 20 cc. of whole urine) into the dorsal lymph space of the Xenopus. Any number of animals were used for this test varying from 5 to 12 according to the technics set up by the different investigators. An extrusion of hundreds of macroscopic eggs after from 6 to 18 hours following the injection was considered a positive reaction for the presence of large amounts of APL hormone.

After receiving personal communications from both Drs. Shapiro and Zwarenstein of Capetown, South Africa, relative to the merits of the Xenopus test for pregnancy, one of us visited the Pregnancy Diagnosis Laboratory of Professor Crew in Edinburgh to see his set-up of the Xenopus for pregnancy testing. The hundreds of healthy animals, and the accuracy of the tests in competent hands together with the rapidity of the test prompted us to continue the investigation with the Xenopus. Accordingly, a small shipment of Xenopus was sent to us from Dr. Crew's laboratory[2] and subsequently one hundred Xenopus arrived from South Africa.

An experiment was undertaken to learn whether the APL hormone of pregnancy urine was actually the responsible factor, and if so, what amounts of the hormone were necessary to produce the positive reactions.

Experimental. The Xenopus were kept at the New York Aquarium for a month prior to being utilized. During that time the animals were well fed twice or three

[1] Read by title before the twenty-fourth annual meeting of the Association for the Study of Internal Secretions, June, 1940.

[2] We wish to thank Professor F. A. E. Crew of the Institute of Animal Genetics of the University of Edinburgh, Scotland, for his encouragement and cooperation in starting this research in the United States.

times weekly with small strips of beef heart, minced steak and chopped liver. At the end of this rest and building-up period we felt that they were accustomed to their new surroundings, in good health and ready to be used. The first experiment was performed in the experimental laboratory of the Jewish Memorial Hospital. Twelve animals were divided into 6 groups of 2 each and placed into 6 2-gallon tanks. Each tank contained 2000 cc. of water and was furnished with a half inch wire mesh one inch above the bottom to prevent the possible eating of any eggs by one or both animals.

The animals in tank *A* were each given injections of 1 cc. of sterile distilled water and were used as the control. The animals in tank *B* were each injected with 10 rat units of a standardized commercial APL hormone preparation derived from pregnancy urine.[3] Similarly, the animals in tanks *C*, *D*, *E* and *F* respectively were injected with 50, 100, 250 and 500 R.U. of the same preparation.

The animals were all injected by the same technic as suggested by Crew, using the dorsum of the animal as the site for the injection and subcutaneously penetrating the dorsal lymph space with care being taken to avoid puncturing the lungs. The tanks were kept at room temperature (20 to 21° C.) and the animals were examined 18 hours following the injections. Tanks *A*, *B* and *C* showed no reaction whatsoever. Tank *D* and *E* in which each Xenopus received 100 and 250 R.U. respectively showed marked reactions with hundreds of ova having been extruded from the cloaca of the animals. The animals in tank *F*, which received 500 R.U. were both dead, probably as a result of over-dosage or interference by preservatives found in larger amounts of the commercial product with increased dosage.

Similar experiments, repeated at the New York Aquarium, demonstrated that the animals receiving 50 R.U. or less of the APL hormone did not react while those receiving 100 R.U. or more reacted strongly.

Results. The anterior pituitary-like hormone when injected in doses of 100 R.U. or more produced marked ovulation in the *Xenopus laevis* within 18 hours after injection. Amounts of the APL hormone of 50 R.U. or less failed to produce any signs of external oviposition. The injection of sterile distilled water produced no reaction.

SUMMARY

Standardized commercial extracts of anterior pituitary-like hormone derived from pregnancy urine when injected into the female *Xenopus laevis* were capable of inducing ovulation and extrusion of hundreds of ova from the South African clawed Toad within 18 hours.

The APL hormone of pregnancy urine is the responsible factor in the Xenopus test for the diagnosis of pregnancy. Amounts of the hormone in excess of 50 R.U. are required to produce the reaction. It is hardly likely that such large amounts of the hormone could be found in normal urines in the quantities injected into the Xenopus.

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[3] The commercial preparation used in these experiments was antuitrin-S prepared by Parke, Davis & Co.

ASSOCIATION NOTICE

ANNOUNCEMENT OF THE ANNUAL MEETING

THE TWENTY-FIFTH ANNUAL MEETING of the Association for the Study of Internal Secretions will be held in Atlantic City, New Jersey, on May 2nd and 3rd, 1941. The Seaside Hotel on the Boardwalk will be the headquarters for registration and for the scientific and business sessions.

The Chairman of the Local Committee is Dr. Matthew Molitch, 705 Pacific Avenue, Atlantic City, New Jersey.

Instructions regarding the reservation of hotel accommodations will be given to all members at a later date.

PRESENTATION OF PAPERS

Members of the Association and other investigators who wish to present papers will please note the following regulations regarding the program for the coming meetings.

1. The title of the paper, and a comprehensive abstract *in quadruplicate*, must reach the President, Dr. Elmer L. Sevringhaus, 1300 University Avenue, Madison, Wisconsin, not later than February 1, 1941.

2. Non-members who present papers will appear on the program "by invitation." Non-members are therefore requested to have their titles and abstracts introduced by a member.

3. The abstracts of papers will be considered by the program committee and the final program will be announced about March 15, 1941.

4. Papers will be limited to ten minutes for presentation and five minutes for discussion.

5. A ten-minute presentation before the Association is obviously designed for the brief and condensed discussion of new investigations, either in laboratory or clinical phases of endocrinology. Work which has previously been published or presented elsewhere, is not desired at such a meeting and consequently, it will be understood that previous publication or oral presentation before any society of national membership will be cause for omitting a paper from the program of the Association.

6. Papers reporting in final form the results discussed before the Association at its Annual Meeting should be submitted to the Editor of ENDOCRINOLOGY or the JOURNAL OF CLINICAL ENDOCRINOLOGY. Such submission is invited. It is to be understood, however, that acceptance of a paper for the program does not obligate either Journal to its acceptance for publication.

You are invited to bring this announcement to the attention of your colleagues and associates who have endocrine research in progress.

NOMINATION OF OFFICERS

The By-Laws of the Association provide that nominations for all elective offices shall be made by a Nominating Committee and forwarded to the Secretary at least sixty days before the annual meeting. The Nominating Committee for the current year, appointed by the President and accepted by the Council, is as follows.

Dr. Philip E. Smith, *Chairman*, College of Physicians and Surgeons, Columbia University, New York City.

Dr. J. B. Hamilton, School of Medicine, Yale University, New Haven, Connecticut.

Dr. H. B. Van Dyke, Squibb Institute for Medical Research, New Brunswick, New Jersey.

The By-Laws also provide (Article V, Section 2) that "Any member of the Association may submit nominations to the Nominating Committee for its consideration." Any nominations which members desire to make should be sent to Dr. Philip E. Smith, 630 West 168th Street, New York City, by February 1, 1941.

The terms of the following officers expire at the time of the annual meeting in Atlantic City in 1941.

President-Elect	Edgar Allen
Vice-President	K. W. Thompson
Secretary-Treasurer	E. Kost Shelton

Council Members for 3 years	Publication Board for 3 years
C. N. H. Long	F. C. Koch
Henry H. Turner	W. O. Nelson
W. O. Thompson	

THE E. R. SQUIBB & SONS AWARD

The E. R. Squibb & Sons award of $1000.00 established in 1939 and first given in 1940, is at the discretion of a special committee of five members of the Association. This award will be given to the investigator or investigators in the United States or Canada for one of the best contributions to endocrinology. Nominations for the Award may be sent to the Secretary by members of the Association, not later than January 15th of the year in which the Award is to be made. Each member has the privilege of making one nomination. Such a nomination should be accompanied by a statement of the importance of the nominee's contributions in endocrinology and by a bibliography of the nominee's most important publications in this field. Five copies should be sent to the Secretary, Dr. E. Kost Shelton, 921 Westwood Blvd., Los Angeles, California, who will transmit the copies to the members of the Committee.

Elmer L. Sevringhaus, *President*
E. Kost Shelton, *Secretary*